Chlorophyll and Green Color Stabilization on Vegetable Homogenates

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Abstract

Several factors influence chlorophyll stability in foods and beverages. This study aims to determine the influence of raw material, heat treatment, pH and food additives on chlorophyll content and color stability of vegetable homogenates. Fully expanded spinach, parsley, broccoli, and lettuce leaves were harvested and immediately processed. Leaf (10%) homogenates were bottled, heat-treated at 90 ºC for 60s, cooled and stored at 20 ºC. Chlorophylls *a* and *b* were measured before and after pasteurization, and during shelf-life. Color was assessed with a colorimeter. Broccoli and parsley, spinach and lettuce had an initial hue angles of 132.8°, 129.0° and 109.8°, respectively. Chlorophyll content was higher in broccoli (171.4 µg mL^{-1}) and lower in lettuce (44.4 µg mL^{-1}). Hue and chlorophyll content decreased in all homogenates during pasteurization. At pH 8.5 the addition of sodium bicarbonate and zinc chloride had a positive effect on retention of chlorophyll and hue of spinach, parsley and broccoli homogenates, with hue increases of 8% in samples treated with sodium bicarbonate and 9% in those with zinc chloride. At pH 4.5 hue decreased 18% in all samples, with accompanying color shift to olive green. During shelf-life, hue and chlorophyll content was better maintained at higher pH in all samples, with higher values registered for broccoli samples, irrespective of additive. Pre-harvest zinc application in lettuce had a positive effect on color of pasteurized samples, with no significant effect in chlorophyll content. However, there was visual evidence of color retention for these samples. Total chlorophylls decreased 19% in all lettuce samples due to heat treatment. While hue, chlorophyll content and visual color were better maintained in samples with pre-harvest treatments during shelf-life, the addition of zinc did not affect these parameters. A significant relationship between sample hue and total chlorophyll content was found, for all vegetable samples in study.

Keywords: broccoli; color; lettuce; parsley; spinach.

Resumo

As clorofilas são pigmentos fotossintéticos presentes nas frutas e hortaliças, e são os principais responsáveis pela cor verde destes produtos hortícolas. As plantas superiores têm geralmente dois tipos de clorofila, a clorofila *a* e a clorofila *b*, que apesar terem uma extrutura semelhante, diferem no substituinte do C-3: a clorofila *a* tem um grupo metilo nessa posição e a clorofila *b* um grupo aldeido. Estas duas estruturas diferem também na sua estabilidade térmica, mais elevada na clorofila *b* que na clorofila *a*. Geralmente a clorofila *a* e a clorofila *b* ocorrem numa proporção de 3:1 em tecidos vegetais verdes, apesar de este racio depender do tecido vegetal e também das condições ambientais durante o desenvolvimento da planta.

A clorofila é geralmente instável durante o período pós-colheita e o processamento de matérias-primas verdes, e durante o processamento térmico a degradação da clorofila segue uma cinética de primeira ordem. Esta é normalmente acompanhada por uma alteração de cor, mudando de verde intenso brilhante para verde amarelado, devido à perda do ião magnésio da estrutura da clorofila, e à sua substituição por dois iões de hidrogénio, formando-se feofitinas. A estabilidade das clorofilas no período de pós-colheita é afetada por vários fatores, como o tipo de processamento, o pH do meio e a temperatura ambiente, bem como a interação com aditivos alimentares normalmente usados durante o processamento. A adição de sais (metálicos e não metálicos) permite estabilizar a clorofila e cor verde de produtos processados, por estabilização da molécula de clorofila ou através da formação de complexos derivados desta, mais estáveis ao calor e com uma coloração verde mais intensa.

Este trabalho teve como objetivo determinar a influência da matéria-prima, do tratamento térmico, do pH e de dois aditivos alimentares, bem como tratamentos foliares de zinco em pré-colheita, sobre a concentração de clorofilas e alterações na cor de homogeneizados de espinafre, salsa, folhas de brócolo e alface. Folhas de espinafre, salsa, brócolo e alface foram processados imediatamente após a colheita e usados para a produzir homogeneizados com 10% de matéria vegetal e 90% de água destilada. Os homogeneizados foram engarrafados em garrafas de vidro, tendo-se procedido ao tratamento térmico das amostras, a 90 °C por 60 s, com posterior arrefecimento até temperatura de armazenamento a 20 °C. O estudo do efeito de aditivos alimentares e do pH do meio foi efetuado nos homogeneizados de espinafre, salsa e folhas de brócolo, com adição de 0.5% de bicarbonato de sódio e de cloreto de zinco a 100 mg kg^{-1} a amostras distintas e ajuste do pH a 4,5, 6,0 e 8,5 antes do tratamento térmico. O tratamento com cloreto de zinco na dose de 200 mg kg^{-1}, foi aplicado foliarmente a alfaces, 3 e 1 dias antes da colheita. A aplicação de 100 mg kg^{-1} de cloreto de zinco aos homogeneizados de alface foi efetuada antes do tratamento térmico. Os parâmetros avaliados foram o pH do meio, o teor de sólidos solúveis totais, parâmetros de cor, a concentração de clorofila *a*, de clorofila *b*, clorofilas totais e carotenóides. Foi também

efetuado o registo fotográfico para reportar variações na cor visivel. Estes parametros foram medidos antes e depois do tratamento térmico, bem como durante armazenamento das amostras a 20 °C por 16 dias para as amostras de espinafre e salsa, e 11 e 18 dias para as amostras de bróculo e alface, respetivamente.

Os homogenizados de salsa e brócolo apresentaram uma tonalidade inicial média de 132.8°, comparado com 129.0° e 109.8° das amostras de espinafre e alface, respetivamente. O teor de clorofila total foi superior no homogeneizado de brócolo (171.4 μg mL^{-1}) e inferior no de alface (44.4 μg mL^{-1}). O tratamento térmico levou a um ligeiro aclaramento de todas as amostras, com consequente aumento do valor de luminosidade. O tratamento térmico reduziu a concentração de clorofilas em 28, 25, 22 e 19% nas amostras de espinafre, salsa, alface e brócolo, respetivamente. A adição de bicarbonato de sódio e cloreto de zinco combinado com o ajuste do a pH 8.5 teve um efeito positivo na manutenção do teor de clorofilas, levando a aumentos médios de tonalidade de 8 e 9%, respetivamente. Foi também observado um aumento da intensidade da cor verde visível das amostras pasteurizadas. Estes aditivos não tiveram efeito positivo na retenção da clorofila nem da cor em amostras pasteurizadas a pH 4.5; neste pH verificaram-se decréscimos médios de tonalidade de 18 e 13%, nas amostras com bicarbonato de sódio e cloreto de zinco, respetivamente, e alteração de cor visível, do verde vivo a verde azeitona.

Apesar de ambos os aditivos alimentares serem eficientes na manutenção da cor e teor de clorofilas a pH 8,5, apenas o bicarbonato de sódio proporcionou boa estabilização destes parâmetros a a pH 6,0. Durante o armazenamento, a tonalidade e o teor de clorofilas foram melhor mantidos a pH 8,5, com melhores resultados observados nos homogeneizados de brócolo, independentemente do aditivo alimentar adicionado. A redução do pH induziu um decréscimo no teor de clorofilas, tonalidade e alteração de cor visivel mais rápido. Os homogenizados vegetais de cor mais intensa antes do tratamento térmico, como as amostras de brócolo, apresentaram melhor retenção de cor após o tratamento térmico, comparado com amostras com cor inicial mais clara e de verde menos intensa, como as amostras de espinafre e salsa, mesmo a pH mais baixo e independentemente do aditivo alimentar.

A aplicação foliar de zinco na fase de pré-colheita da alface teve um efeito positivo na preservação da cor visível das amostras pasteurizadas, mostrando evidência do efeito do zinco na retenção da cor das amostras. O valor de tonalidade mais alto sem tratamento térmico foi registado nas amostras com aplicação de zinco pré-colheita (115°), comparado com amostras sem aplicação de zinco pré-colheita (111°). O tratamento térmico originou perdas médias de clorofila *a* de aproximadamente 27% em todas as amostras. As perdas de clorofila *b* com o tratamento térmico foram de 13% nas amostras CT_ZC, 9% nas amostras ZC_CT e 8% nas amostras de CT_CT. O teor de clorofila total decresceu aproximadamente 22% em todas as amostras, excepto nas amostras de ZC_ZC, onde o decréscimo foi de 16%.

Durante o armazenamento dos homogeneizados de alface, a tonalidade, o teor de clorofilas, e a cor visivel das amostras foram melhor mantidos nos homogeneizados com tratamentos pré-colheita foliares de zinco. Em todas as amostras foi observado um decréscimo inicial, seguido de uma manutenção, do valor de tonalidade e teor de clorofila, até ao dia 18. No entanto, as amostras sem aplicação foliar de zinco tiveram um decréscimo mais acentuado comparativamente às amostras sem aplicação de zinco: perdas de clorofila de 40% e 34% em amostras CT_CT e CT_ZC comparado com 26% de perda em amostras ZC_CT e ZC_ZC, e perdas de tonalidade de 109,3° para 94,7° e 112.2° para 95,3° em amostras CT_CT e CT_ZC, respetivamente, comparado com 115.5 para 103.6 e 114.5 para 103.6 em amostras ZC_CT e ZC_ZC, respetivamente. A adição de zinco após a produção dos homogeneizados não teve nenhum efeito na manutenção destes parâmetros durante o shelf-life.

Foi encontrada uma correlação linear significativa entre a tonalidade e a clorofila para as amostras de homogenizados vegetais em estudo: 0,9 para as amostras de alface e brócolo, 0,8 para as amostras de espinafre e 0,7 para as amostras de salsa.

Palavras-chave: alface; brócolo; cor; espinfre; salsa.

Abbreviation List

1-MCP	1-Methylcyclopropene
Car	Carotenoid
Chl-a	Chlorophyll *a*
Chl-b	Chlorophyll *b*
Chl (a+b)	Total chlorophyll
Chl (a/b)	Ratio between chlorophyll *a* and chlorophyll *b*
CT	Control sample
HT	With heat treatment
MCS	Metal chelating substance
MRP	Magnesium-releasing proteins
NHT	Without heat treatment
LDS	Least significant difference
SB	Sodium bicarbonate
SSC	Soluble solid content
ZC	Zinc chloride

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Chapter 1

Introduction

Color is a major food quality attribute. Color changes in fresh and processed foods provide clues regarding the level of freshness and overall quality. The green hue is characteristic of several raw foods, including leafy, stem (e.g. green asparagus), inflorescence (e.g. broccoli), and immature fruit (e.g., cucumber and snow pea) vegetables as well as some fruit (e.g. the peel of Granny Smith apple, unripe olives, and kiwifruit flesh). Chlorophylls are the pigment that confer green color to foods of plant origin. Chlorophylls are the most widely distributed plant pigments, with the biological role of harvesting light energy to be used in photosynthesis (Belitz et al., 2009).These pigments are particularly unstable under most conditions relevant for food processing and preservation (Koca, et al. 2008).

Natural developmental changes, including senescence and ripening, influence chlorophyll content of fresh green produce. In most vegetables, senescence has a negative effect on the visual appearance with color as a determinant variable. The loss of green color and the color change to yellow or brown hues is due to chlorophyll degradation and uncovering of preexistent or *de novo* synthesized carotenoid pigments (Hörtensteiner & Kräutler, 2011). Chlorophyll degradation in plant tissues occur during natural leaf senescence and fruit ripening, and as a response to various biotic and abiotic stresses, including temperature, humidity and light conditions (Hörtensteiner, 2013).

Green raw materials can be processed by several means including canning, freezing, dehydration, brines, pickling, and transformation into purees, pulps, and juices. The industrial

processes involved include size reduction, heat treatments, lowering temperature, exposure to acid and salty environments, leading to extreme changes in tissue structure and creating novel conditions for biochemical and chemical changes in the pigments. Some of these processing techniques are very detrimental to chlorophylls, and stabilization of green color remains a challenge. Processing of vegetable purees and homogenates implies total destruction of tissue structure and cellular compartmentation of the raw material and oxygen incorporation during processing. In addition, heat treatments and acidification used as standard practice to assume microbial stability and safety also hasten chlorophyll breakdown. Combined, these factors pose major challenges to the retention of green color of food matrices during shelf-life.

Coloring food additives are available to modulate color in processed foods. Among the 41 (Food Standards Agency, 2016) coloring additives currently authorized in the European Union, only three are green: chlorophylls and chlorophyllins (E 140); cooper complexes of chlorophylls and chlorophyllins (E 141) and Green S (E 142) (Regulation (UE) n° 231/2012). The scarce possibilities to modulate green - a color that is so prevalent in nature – are demonstrated by this limited number of green coloring additives, and the “clean label” trend that imposes further limitations to their use in foods and beverages.

1.1. Objectives

The work presented in this thesis aimed to understand and quantify the effects of different techniques on chlorophyll and green color stability in liquid vegetable matrices. Specific objectives of the study were to evaluate the effect on green color and chlorophyll content of the following control variables used in the food industry:

- Raw materials (spinach, parsley, broccoli and lettuce);
- Food additives (sodium bicarbonate and zinc chloride);
- pH.

Interactions between the effect of food additives and pH on color and chlorophyll content were also addressed, as well as the possibility of pre-harvest zinc applications to stabilize the green color and chlorophyll content of pasteurized vegetable homogenates.

1.2. Thesis structure

This thesis is organized in 5 chapters. After the introduction to the scope of the research subject (chapter 1), chapter 2 addresses the chlorophyll pigments, the factors affecting their stability *in planta* and in cell free food matrices, and their degradation kinetics as related to color. The variation of color and chlorophyll content on different raw materials is briefly summarized. This chapter also reviews the relevant information about the effect of industrial food processing methods on green color and chlorophyll. Chapter 3 describes the materials and methods used in the studies, and chapter 4 presents and discusses the results of the experimental work. Chapter 5 concludes and point out directions for future work on issues raised in the thesis or that remained inconclusive.

Chapter 2

Literature Review

This chapter introduces some fundamental concepts related to chlorophyll pigments and the green color of vegetables and vegetable-derived foods and beverages. The molecular structure of chlorophyll and the main changes that it undergoes when degraded and the kinetics of degradation are reviewed, as well as the factors contributing to its stability, in green tissues *in vivo* and in nonliving food matrices. The range of chlorophyll content in vegetable raw materials and the changes in color in the fresh product is also addressed. Then, the influence of processing conditions, such as temperature and the duration of heat treatments, pH and additives, as related to chlorophyll and color of processed vegetable matrices, is discussed. Additionally, this chapter also reviews less common or emerging food processing methods, thermal and non-thermal, and their influence on chlorophyll and color stability.

2.1. Chlorophylls: the structures and reactions of the green pigments

2.1.1. Molecular structures and spectral properties

Chlorophylls are derivatives of the tetrapyrrole phorbin containing a magnesium ion. Higher plants have two types of chlorophylls, chlorophyll *a* and chlorophyll *b* (Figure 1). Although similar in the main structure, these compounds differ in the substituent on the C-3 carbon position: chlorophyll *a* has a methyl group whereas chlorophyll *b* has a formyl group in that position (Belitz et al., 2009).

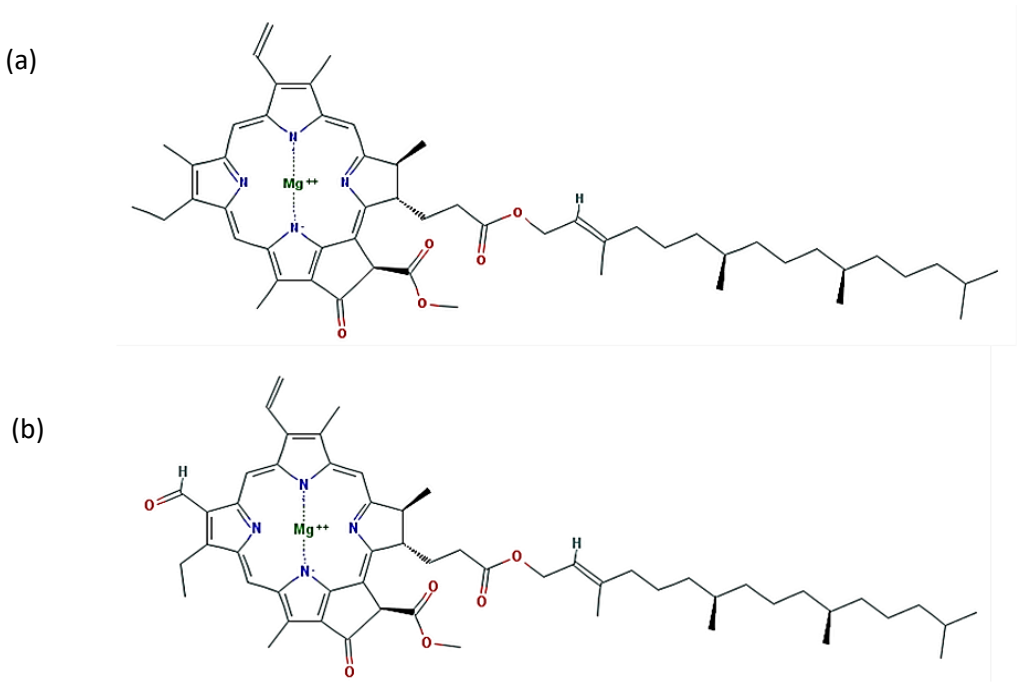


Figure 1- Chemical structure of chlorophyll a (a) and chlorophyll b (b). Structures retrieved from PubChem: Chlorophyll a (PubChem CID 12085802); Chlorophyll b (PubChem CID 6450186).

Chlorophyll *a* is the primary photosynthetic pigment in all higher plants, algae, and cyanobacteria, accompanied by chlorophyll *b* at lower concentrations. Chlorophyll *b* absorbs strongly in the 450-480 nm range, filling the gap in the absorption spectrum of chlorophyll *a* (Gross, 1991). Chlorophyll *b*, however, does not seem essential for survival of higher plants since viable mutants deficient of chlorophyll *b* are known (William & Huner, 2009). Diatoms, dinoflagellates, and brown algae, in addition to chlorophyll *a* also contain chlorophyll *c* (Manning & Strait, 1943), while cyanobacteria, photosynthetic prokaryotes, contain other chlorophyll structures, named chlorophyll *d* and *f* (Miyashita et al., 2014).

The structural differences between chlorophyll *a* and *b* (Figure 1) alter the color of these molecules: chlorophyll *a* is blue-green and chlorophyll *b* is yellow-green. The two structures

also differ in their thermal stability, higher in chlorophyll *b* than in chlorophyll *a* (Weemaes et al., 1999).

Chlorophyll *a* and *b* usually occur in green plant tissues in the proportion of 3:1 (Gaur et al., 2006), although this ratio vary slightly among plant tissues (Belitz et al., 2009) and can be affected by environmental factors during leaf development. Leaves exposed to sunlight have higher chlorophyll *a* to chlorophyll *b* ratio (3.0 to 3.8) than shade leaves (2.4 to 2.7). The higher proportion of chlorophyll *b* in shade leaves makes them more effective in harvesting low intensity light (Lichtenthaler & Buschmann, 2001).

2.1.2. Chlorophyll location and catabolism in plant cells

Chlorophylls are lipid-soluble substances located in the thylakoid membranes of the chloroplasts. The breakdown of chlorophylls *in planta* occurs during the developmental processes of leaf senescence and fruit ripening and also in response to biotic and abiotic stress (Hörtensteiner, 2013). Chlorophyll catabolism is mediated by enzymes whose expression is regulated. Expression of genes coding enzymes required for chlorophyll degradation occur during senescence and as a response to environmental cues inducing chlorophyll catabolism (Hörtensteiner, 2013).

In vivo chlorophyll catabolism starts in the chloroplast, where the pigment is located, but downstream catabolites pass though the cytosol in transit to the vacuole where the final colorless breakdown products accumulate.

Chlorophyll catabolism is accompanied by a shift in color from brilliant green to olive brown in processed foods, and to a wide variety of colors like yellow, brown or orange, in senescent tissues (Heaton & Marangoni, 1996). While in senescent tissues the color change is attributed to the unmasking of yellow carotenoids and, in some cases, to the synthesis of red anthocyanins (Christ & Hörtensteiner, 2014), the olive brown color results from the loss of magnesium in the chlorophyll structure and its replacement by hydrogen to form pheophytins (Figure 2) (Schwartz & Von Elbe, 1983).

Comprehensive and updated reviews of the biochemistry of chlorophyll catabolism are available (Hörtensteiner, 2006; Matile et al, 1999). Chlorophyll catabolism in planta can be summarized as indicated in Figure 2.

In brief, the metabolic breakdown of chlorophyll molecules can follow two pathways. One is the formation of bright green chlorophyllide, due to the removal of the phytol chain from the chlorophyll structure catalyzed by the enzyme chlorophyllase, which is then converted into pheophorbide when the magnesium ion is removed. The second pathway involves the formation of olive brown pheophytin due to loss of Mg^{2+} by action of the enzyme Mg-

dechatalase and subsequent removal of the phytol chain, resulting in the formation of pheophorbide.

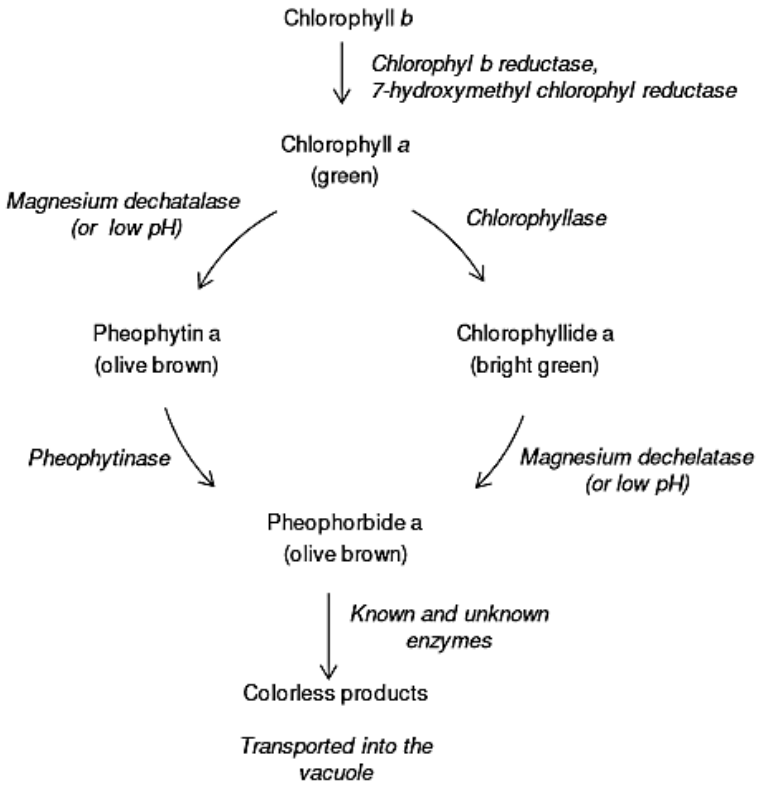


Figure 2 - Simplified scheme of chlorophyll breakdown pathways in planta (Løkke, 2012).

Subsequent reactions convert this molecule into colorless products by cleavage of the porphyrin ring, where they are transported from the chloroplasts into the vacuole for further degradation. At low pH the magnesium of the porphyrin ring is replaced by hydrogen ions to generate pheophytin (Zheng et al., 2014). In this case there is no formation of colorless catabolites due to cell death; the inhibition of further degradation of pheophytin maintains the tissue color olive brown.

Chlorophyll degradation in cell free systems

Chlorophyll degradation in living plant tissues does not occur in cell free matrices, like purees or juices. However, the chlorophyll status in the raw material is likely determinant for the stability of green color in processed products. Two types of chlorophyll breakdown mechanisms have been described: Type I, previously described, involves very controlled and relatively well described transformations, and type II breakdown, far less controlled and mediated by oxygen radicals. A characteristic of type II breakdown is that it happens when the membrane systems within the cell are disrupted prior to their initiation, i.e. when membrane

breakdown and loss of sub-cellular compartmentalization occurs (Toivonen & Brummel, 2008), due to senescence or food processing, as in the case of fresh-cut products.

Chlorophyll retention is related to the preservation of chlorophyll-binding proteins, as well as inhibition of the catabolism of thylakoid lipids, since chlorophyll degradation is the first step in thylakoid degradation. During processing operations, such as chopping, cooking or freezing of plant material, released intracellular acids and enzymes contact with chlorophyll-protein complexes (Heaton & Marangoni, 1996). This contact, combined with the physical damage to the plant tissue, initiates chlorophyll degradation.

Most processed foods and beverages are nonliving matrices with no metabolic activity. If these matrices are enzymically active, e.g., during homogenization, enzyme catalyzed reactions may mediate chlorophyll degradation. When the enzymes in the food matrix have been inactivated, e.g., by a heat treatment, chlorophyll degradation occurs via chemical reactions not catalyzed. In contrast, minimally processed foods maintain metabolic activity and thus chlorophyll degradation is affected by both environmental and genetic factors. However, further studies are required to understand how chlorophyll and chlorophyll-binding proteins are affected by different processing conditions (Heaton & Marangoni, 1996).

2.2. Chlorophyll content in raw materials and its changes

2.2.1. Chlorophyll content

Chlorophyll content in raw materials varies widely and depend on (i) type of vegetable; (ii) growing conditions; (iii) stage of maturity at harvest; and (iv) postharvest conditions.

The range of chlorophyll content of various fruits and vegetables is exemplified in Table 1. These values, however, should be taken as indicative since there is a wide variation among cultivars of the same species and differences depending on the stage of maturity and growing conditions (Ferruzzi & Schwartz, 2001). For example, Table 1 illustrates a 23-fold variation in total chlorophyll between cabbage and kale leaves, vegetables belonging to the same species *Brassica oleracea*.

Table 1 - Chlorophylls a and b in vegetables and fruits (adapted from Belitz et al., 2009).

Food	Chlorophyll *a* (mg kg^{-1} fresh weight)	Chlorophyll *b* (mg kg^{-1} fresh weight)	Total chlorophylls (mg kg^{-1} fresh weight)
Gooseberry	5	1	6
White cabbage	8	2	10
Kiwi	17	8	25
Cucumber	64	24	88
Green peas	106	22	128
Green bell pepper	98	33	131
Green beans	118	35	153
Spinach	946	202	1148
Parsley	890	288	1178
Kale	1898	406	2304

2.2.2. Factors affecting chlorophyll content in planta

Physiological age of leaves directly influences chlorophyll concentration and color. Maximum chlorophyll content occurs when leaves are fully expanded and before the onset of senescence. This is illustrated, for instance, in kale (*Brassica oleracea* var. *acephala*) leaves developed under controlled environmental conditions and analyzed at 5 stages of development: young (<1 week), immature (1–2 weeks), mature (2–3 weeks), fully developed (3–4 weeks) and senescing (>4 weeks). Levels of chlorophyll *a* and *b* increased as the leaves developed, reached a maximum at 2-3 weeks and declined thereafter (Lefsrud et al., 2007).

Among the environmental growing conditions that affect chlorophyll content, light plays as important role, not only on total chlorophyll content but also on the ratio between chlorophylls *a* and *b*. Whenever light availability is not limiting, increased light intensity reduces leaf chlorophyll content. This response has been reported for several species. However, interactions among environmental factors play a role. Under high light intensity, higher soil fertility increases leaf chlorophyll concentration, as has been shown beech (*Fagus sylvatica*) seedlings (Minotta & Pinzauti, 1996).

2.2.3. Effect of postharvest handling before processing

Maximum chlorophyll content is attained in fully expanded leaves prior to the onset on senescence and optimized growing conditions such as limited light and proper fertilization can enhance chlorophyll content in a given raw material.

However, chlorophyll senescence begins immediately after harvest. Therefore, the conditions and the time mediating between harvest and processing are likely to affect the content of chlorophyll and the status of the pigments, even though the apparent green color remains unchanged.

A model for postharvest color changes in green vegetables has been proposed (Figure 3), suggesting that these are biphasic and the durations of these phases is differentially affected by exogenous factors (Vasconcelos & Almeida, 2003).

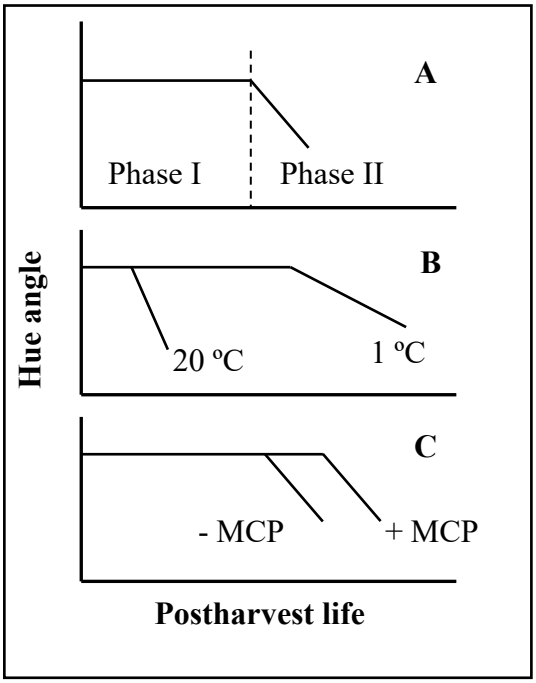


Figure 3 - Schematic representation of the effect of temperature and 1- MCP on color development in broccoli. (A) biphasic pattern of color development, (B) effect of temperature, and (C) effect of 1-MCP. (Vasconcelos & Almeida, 2003).

This biphasic pattern has been observed in several green organs, including leaves (lettuce and cilantro), inflorescences (broccoli) and fruit (cucumber), suggesting that is a general phenomenon related to chloroplast senescing after harvest in green vegetables. During phase I, color remains green but chlorophyll degradations is likely to occur, considering the modulation of the duration of its phase by temperature or ethylene. During phase II, yellowing is evident and the rate of color change is determined by temperature. If this hypothesis proves to be true, it is expected that juices prepared from green leaves at the end of phase I, although green and unable to be distinguished in quality control, are likely to have degraded chlorophylls that will not be stable after processing.

2.3. Chlorophyll degradation kinetics

The use of kinetic models to describe quality changes, namely color or chlorophyll content, provide a means to improve the design of food processes and control systems (Gaur et al., 2006). Kinetics parameters like the rate constant and activation energy provide a quantitative means to assess the effect of process variables and to predict changes in chlorophyll content or in hue.

Chlorophyll degradation in cell-free systems is usually described by a first order kinetic model (Ahmed et al., 2013; Canjura et al., 1991; Gaur et al., 2006; Nisha et al., 2004), represented by equation 1 (Gaur et al., 2006).

$$ln\,{}^{C}/_{C_0} = -\,kt \tag{1}$$

where *C* is the pigment concentration at time t, C_0 denotes measured pigment concentration at zero time, *k* is the rate constant and t is the time.

The temperature dependence of chlorophyll degradation during storage follows a first order kinetic model, which combined with the Arrhenius equation, results in the expression of equation 2 (Martins & Silva, 2002).

$$\frac{C}{C_0} = exp\left(-k_{ref}.e^{-\frac{E_a}{R}\left[\frac{1}{T}-\frac{1}{T_{ref}}\right]}.t\right) \tag{2}$$

where C is the concentration of chlorophyll *a* or *b* at time t, C_0 is the initial concentration, k_{ref} the kinetic rate at the absolute reference temperature T_{ref}, T is the absolute temperature (K), R the universal gas constant and E_a the Arrhenius activation energy.

Fractional conversion is also employed for kinetic data reductions, and for irreversible first-order reaction kinetics, the rate constant at constant temperature can be determined through fractional conversion, *f*. The fractional conversion (*f*) for green color loss can be defined by equation 3 or by equation 4 (Ahmed et al., 2013).

$$f = \frac{[(-a_0)-(-a)]}{[(-a_0)-(-a_\infty)]} \tag{3}$$

$$\ln(1-f) = ln\frac{[(-a_0)-(-a)]}{[(-a_0)-(-a_\infty)]} = -\mathrm{kt} \tag{4}$$

where, a_0 is the green color value at time zero, -*a* is the green color value at time t and $-a_\infty$ is the color value at infinite time. For first-order reaction, (1 - *f*) vs *t* is linear, and the rate constant (*k*) is in the negative of the slope.

A first order reversible kinetic model (fractional) adequately described the evolution of the color parameter a with storage time (equation 5), assuming that initial and final color coordinates were not dependent of storage temperature and that color changed only as consequence of pheophytisation.

$$\frac{C - C_{eq}}{C_0 - C_{eq}} = exp\left(-k_{ref}.e^{-\frac{E_a}{R}\left[\frac{1}{T}-\frac{1}{T_{ref}}\right]}.t\right) \quad (5)$$

where C is the a at time t, C_0 the initial value of a, C_{eq} is the a at infinite storage time (all chlorophyll converted into pheophytin), and k_{ref} the kinetic rate at the reference temperature.

2.4. Alterations in chlorophyll

2.4.1. Enzymatic reactions

Chlorophyllase is the main enzyme responsible for the degradation of chlorophyll *in vivo*. This enzyme catalyses the hydrolysis of chlorophyll to chlorophyllide and phytol (Chen et al., 2012), and its Mg-free derivatives (pheophytins) to chlorophyllides and pheophorbides, respectively (von Elbe, 2000). Although chlorophyllase has a preferential effect on chlorophyll *a*, it also accepts chlorophyll *b* and pheophytins as subtracts (Chen et al., 2012). This enzyme is active in aqueous, ethanoic, and water and alcohol solutions, as well as acetone solutions (von Elbe, 2000).

Chlorophyllase optimal temperature is 30 °C to 35 °C (Ihl et al., 1998) and is inactivated at temperatures above 85 °C, retaining half of its maximum activity at this temperature. Differences in temperature tolerance have been reported for chlorophyllase from different sources. Wheat chlorophyllase are more heat-stable than other chlorophyllases and retain optimal activity at temperatures up to 75 °C (Arkus et al., 2005).

The stability of purified chlorophyllase from green rye seedlings was temperature dependent in the presence of acetone: at 4 °C, this enzyme was stable at pH 6-9 in the presence of 30% acetone, while at 30 °C it was not stable above 10% acetone content (Tanaka et al., 1982). Olive chlorophyllase extract had an optimum pH at 8.5 in an acetate-phosphate-borate buffer (Mínguez-Mosquera et al., 1994) for all substrates used (chlorophylls *a* and *b* and pheophytins *a* and *b*), while chlorophyllase extract of *Ailanthus altissima* (tree-of-heaven) had an optimum pH of 4.5 using pheophytin as a substrate (McFeeters et al., 1971).

Chlorophyllase is inhibited by metallic ions such as Mg^{2+}, Hg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+} and Fe^{3+}. Mg^{2+} and Fe^{3+} have the least inhibitory effect due to the fact that both closely relate to the chlorophyll biosynthesis pathway, and may be present in an *in vivo* environment. The highest inhibitory effect is attributed to Hg^{2+} and Fe^{2+} (Hornero-Méndez & Mínguez-Mosquera, 2001).

In the early stages of chlorophyll degradation, conversion from chlorophyllides to pheophorbides is known to be catalized by the action of the enzyme Mg-dechelatase (Suzuki & Shioi, 2002). This enzyme is located in the tylacoids and is found at high levels in pre-senescent chloroplasts (Suzuki & Shioi, 2002), and has been reported as a heme iron-containing enzyme.

The release of the central magnesium ion can be catalysed by two types of activities: Mg-dechelatase can either be catalysed by a protein, designated as MRP (Mg-releasing proteins), or by a heat stable, low molecular mass compound named MCS (metal chelating substance) (Büchert et al., 2011).

Substract specificity accounts to the difference in activity of MRP and MCS. The metal chelating substance is active with chlorophyllin (artificial substrate of chlorophyll) and chlorophyllide (natural chlorophyll substrate), while Mg-releasing proteins are only active with chlorophyllin (Hörtensteiner & Kräutler, 2011). When trying to identify the activity of the true Mg-dechelatase it is necessary to use the native substrate, i.e. chlorophyllide, in the presence of metal chelating substances (MCS). Enzymes such as glutathione S-transferases and peroxidase were identified as one of various Mg-releasing proteins (Kunieda et al., 2005).

Magnesium dechelatase in fully yellow banana peel was stable at a temperature range of 30-70 °C, and determined optimum temperature was 40-50 °C (Yang et al., 2007). Increasing pH led to the gradual increase of mg-dechelatase activity, with optimum pH at a range of 6.5 to 9.5 (Yang et al., 2007). The activity of this enzyme also varies with the ionic strength of the extraction medium. Mg-dechelatase activity from strawberry extracted with a low ionic strength solution increased continuously with temperature (temperature ranged from 20 to 80 °C), while mg-dechelatase extracted with a high ionic strength solution had an optimum temperature of 50 °C (Costa et al., 2002).

2.4.2. pH

The structure of the chlorophyll molecule is highly influenced by the pH of the food matrix, which has a strong influence on degradative reactions and color stability. Arguably, pH is the major cause of green vegetable discoloration during processing (Aamir et al., 2013; Gaur et al., 2006).

Vegetable raw material differ in their bulk pH (Table 2), and these values must be considered whenever chlorophyll stability is an objective in a food or beverage product.

Table 2 - pH values of selected green vegetables (adapted from Aamir et al., 2013)

Vegetable	pH
Cabbage	5.2-6.3
Broccoli	5.2-6.3
Artichoke	5.38-6.89
Asparagus	5.5-5.8
Cucumber	5.6
Lettuce	6.0-6.5
Spinach	6.0-7.0
Brussels sprout	6.5
Leek	6.5-7.0

In addition to the pH of raw materials, pH is often altered in foods and beverages due to formulation, process or to improve microbial stability. The pH does not remain constant during thermal processing of vegetables; the extent of pH changes varies with the type of vegetable, the initial pH and the time-temperature history of the vegetable of interest (Ryan-Stoneham & Tong, 2000).

The degradation kinetics of chlorophyll in pea puree as a function of pH (5.5, 6.2, 6.8 and 7.5) shows that chlorophyll stability is higher at higher pH and can be better maintained if the pH is held constant during the shelf-life (Ryan-Stoneham & Tong, 2000). This pH effect on chlorophyll stability is observed in several other matrices. In a heat-treated homogenized puree of Thompson seedless grapes, the neutral and alkaline pH values (pH 6, 7, 8, 9 and 10) result in a slightly higher relative chlorophyll pigment retention than acidic values of pH 2, 4 and 5 (Zheng et al., 2014). However, the retention of chlorophyll was not linearly correlated with the pH in the range 2 to 10: maximum chlorophyll retention was observed at pH 3 and the lower concentration at pH 5. The reason for this trend is not known, but differential stability of different pigments (e.g. chlorophyll *a* and *b* and co-pigmentation effects) may account for it.

The rate constants of green color and chlorophyll degradation in blanched green peas decreased with increasing pH over the range 5.5 to 7.5 (Koca et al., 2007). Although chlorophyll *a* degraded faster than chlorophyll *b* at all pH values for each temperature applied (70, 80, 80 and 100 °C), chlorophyll *a* is more susceptible to thermal degradation than chlorophyll *b* in acidic conditions (Koca et al., 2007).

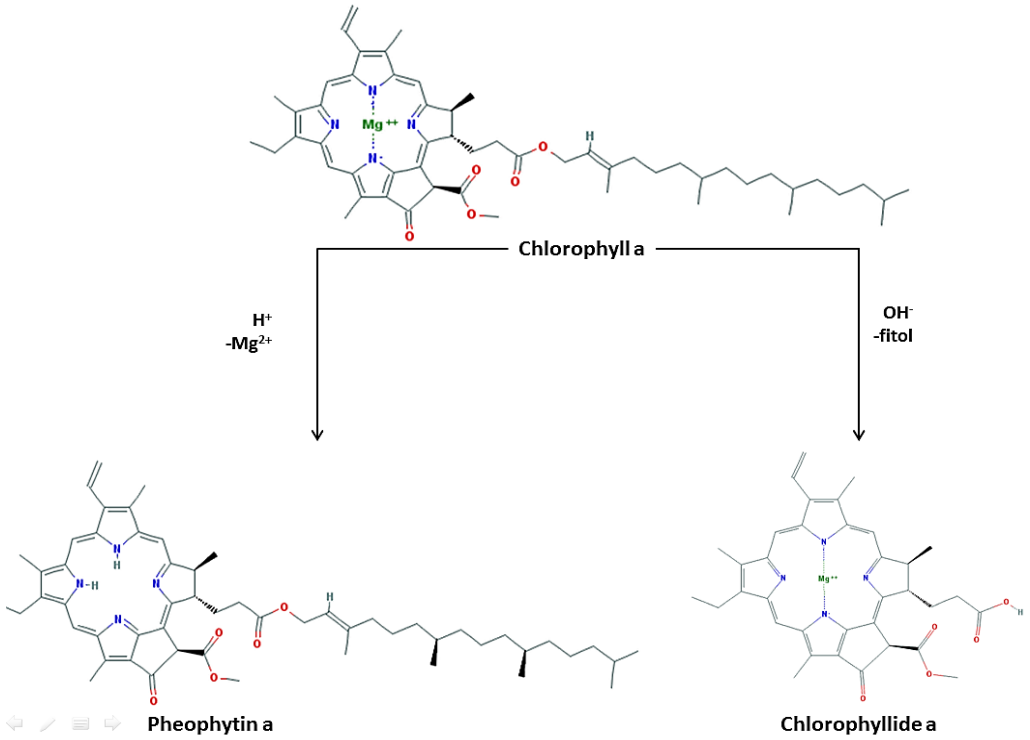


Figure 4 - Overall reaction scheme for the hydrolysis (H^+) and alkaline reaction (OH^-) with chlorophyll (adapted from Streit et al. 2005). Structures retrieved from PubChem: Chlorophyll *a* (PubChem CID 6433192), Pheophytin *a* (PubChem CID 5459387), Chlorophyllide *a* (PubChem CID 439664).

Mg^{2+} replacement by H^+ in tetrapyrrole ring of the chlorophyll molecule is faster under acid conditions and when hydrophobic acids are used in the buffer solution (Tijskens et al., 2001). The rate of color degradation is linearly correlated to the concentration of hydrogen ions over the pH range 3 to 8. However, in tissues, more hydrophobic acids migrate easier though the cell membranes into the chloroplasts, explaining the higher rates of Mg^{2+} replacement when hydrophobic acids, such as phthalic and benzoic acid, are used (Tijskens et al., 2001).

In plant tissues, pH values > 7 stabilize chlorophylls due to the effect of positive ions that decrease membrane permeability and lead to an equilibrium between positive and negative charges, decreasing chlorophyll degradation (von Elbe, 2000), while in cell free matrixes, the chlorophyll molecules can undergo oxidation of the isocyclic ring and de-esterification of the phytol tail, leading to the formation of chlorophyllide (Zheng et al., 2014). In acidic media magnesium in the porphyrin ring is replaced by hydrogen ions (Zheng et al., 2014), converting chlorophylls to pheophytins, with consequent changes in original color, from green to olive green (Andrés-Bello et al., 2013) (Figure 4).

2.4.3. Effects of non-metallic salts

Several ions have positive effects on chlorophyll stability and color retention in foods. The corresponding salts can be used as additives to improve green color. Among these are two sodium salts – sodium chloride and sodium bicarbonate – and the metal ions magnesium, zinc and copper.

Sodium chloride

Sodium chloride is reported to stabilize chlorophylls and the green color of food products (Haisman & Clarke, 1975), although the mechanism is not unequivocally established (Nisha et al., 2004). Sodium chloride (1% and 2%) added to spinach puree heated at 50 to 120 °C improved the stabilization of green color (Nisha et al., 2004). Similarly, sodium chloride (1 and 2%) added to fluted pumpkin (*Telfairia occidentalis*), a green fruit, submitted to heat treatment, also reduces the chlorophyll degradation rate (Ezekiel et al., 2011).

Sodium bicarbonate

Sodium bicarbonate is effective in maintaining green color of heat processed vegetable products.

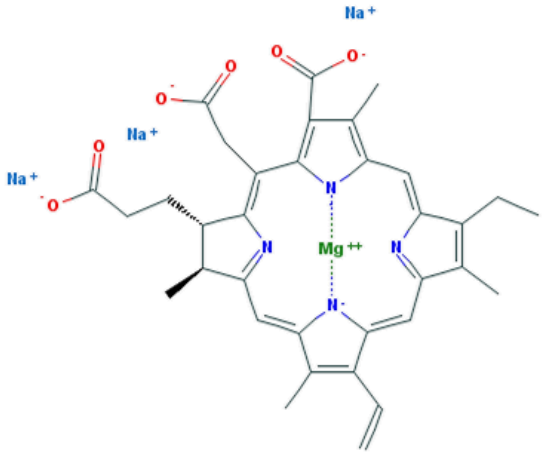


Figure 5 – Molecular structure chlorophyllin sodium complex (PubChem CID 92043294).

Sodium bicarbonate reacts with chlorophyll, displacing the phytyl and methyl groups, forming a bright green water soluble chlorophyllin (Figure 5) (Srilakshmi, 2003). In addition, sodium bicarbonate increases the medium pH which, by itself, improves chlorophyll stability. The sodium salt of chlorophyllin confers an intense greenness to the food matrix, often perceived as “artificial”.

2.4.4. Metallic salts

Metal ions can influence chlorophyll stability and green color. “Regreening” is a process of formation of metallo-chlorophyll complexes due to reaction between chlorophyll derivatives formed during heat treatments and metallic ions that take the place of magnesium in the original chlorophyll molecule (La Borde & Von Elbe, 1994). These complexes are not only more heat and acid resistant than naturally occurring magnesium chlorophyll complexes (Gaur et al., 2006), but can restate the green color (Zheng et al., 2014). Pheophythin is less reactive than other chlorophyll derivatives like pyropheophytin and pheiphorbide, forming metallo complexes more slowly. Therefore, the formation of green metallo complexes of chlorophyll derivatives is favored by increased process time and temperature, which results in the formation of pyropheophythin (Yin et al., 2007).

This process has been considered a good means to preserve the color of canned green vegetables (La Borde & Von Elbe, 1994), since the metallo-chlorophyll complexes not only regreen the product but are also more stable to heat and acid conditions than chlorophyll (Zheng et al., 2014).

Magnesium

Magnesium carbonate improves the retention of chlorophyll in the spinach puree treated at high-temperature short-time (HTST) at 150 °C but the effect is not persistent during storage (Gaur et al., 2006). The formation of hard white crystals of magnesium-ammonium-phosphate during storage of puree with added magnesium carbonate (Gaur et al., 2006) can hinder its use in certain food and beverage matrices, such as purees, juices and concentrates.

However, carbonates are often used as alkalizing or alkalizing-buffer agents and number of patents have noted its industrial application in canned vegetables (Hekal & Erlandson, 1984; Gieseker, 1949).

In addition to magnesium, zinc and copper are metals that have been used to attempt to preserve chlorophyll and green color.

Zinc

Zinc is the most commonly used and studied metal in relations to the preservation of green color (Guzmán et al., 2002; La Borde & Von Elbe, 1990, 1994; Ngo & Zhao, 2007; Tonucci & Von Elbe, 1992).

Zinc ion reacts with chlorophyll to form zinc-chlorophyll complex (Figure 6). Zinc can also form complexes with some chlorophyll derivatives, and Zn-pheophythin *a* and *a’*

complexes were found to contribute to the green color of processed pear peels (Ngo & Zhao, 2007). The complexation reaction between zinc and chlorophyll *a* derivatives follows a second order kinetics (Tonucci & Von Elbe, 1992).

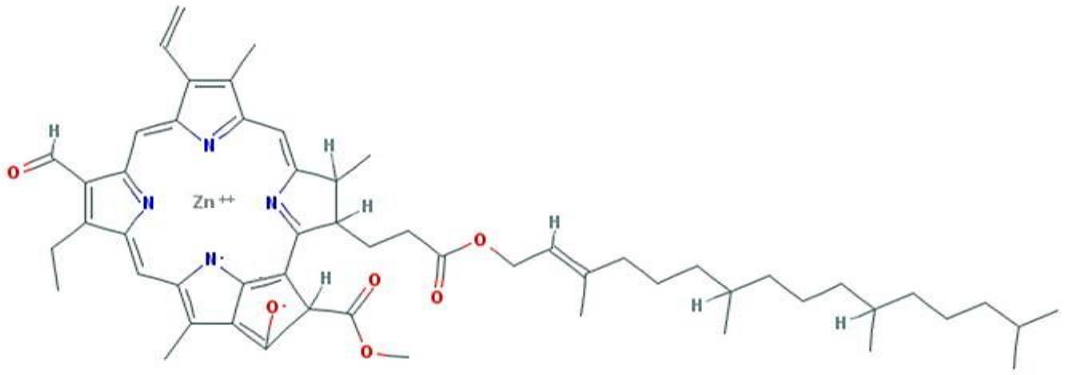


Figure 6 - Molecular structure of zinc pheophytin b (PubChem CID 6442142).

The formation of zinc-chlorophyll complexes depends of various factors, like zinc concentration, pigment concentration (La Borde & Von Elbe, 1990), and also of the chlorophyll derivate concentration (Scipioni, Argüello, & Schmalko, 2010). Studies with blanched spinach, romaine lettuce, and Chinese cabbage showed that significant color preservation require 200-300 µg g^{-1} of zinc chloride in the solutions (Min et al., 2004).

In green peas, zinc-pheophytins complexes are the dominant green compound formed during heating; in the presence of added zinc the concentration of zinc-pheophytins complexes is 242% higher than that of present in peas heated in water containing no added zinc (Ngo et al., 2007).

Copper

Copper salts are 30 times more reactive than zinc salts in heated spinach puree. Competition between zinc and copper ions in the formation of complexes exist. Even in relatively small amounts of copper salt present in the puree, greatly reduced the amounts of zinc complexes formed (Jones, et al. 1977). Copper is very effective in stabilizing chlorophyll, and the Cu-chlorophyll and Cu-chlorophyllin complexes are stable and authorized coloring agents (E141). However, the use of Cu^{2+} by the food industry is limited by its toxicity (Scipioni et al., 2010).

pH effect on metal complexation with chlorophyll

pH plays a central role in metal complexation with chlorophyll and its derivatives. For instance, magnesium chloride ($MgCl_2$), zinc chloride ($ZnCl_2$), copper sulfate ($CuSO_4$) and

potassium chloride (KCl) added to grape puree as sources of the metallic ions Mg^{2+}, Zn^{2+}, Cu^{2+} and K^{+} (1 g L^{-1}), respectively, reduced relative chlorophyll retention at pH 3 but not at pH 4 (Zheng et al., 2014). At pH 4 an increase in the retention of chlorophyll was observed for all salts in the following order: $Cu^{2+} > Mg^{2+} > K^{+} > Zn^{2+}$ (Zheng et al., 2014).

2.4.5. Photodegradation

Chlorophylls are located inside chloroplasts and are protected against various radicals produced during photosynthesis by lipids, proteins and carotenoids in the thylakoid membranes (Rontani et al., 1995). Tissue disruption during food processing, pigment extraction, but also natural plant senescence, remove these protections, exposing chlorophyll to photodegradative reactions (von Elbe, 2000). Singlet oxygen ($^1O_2$) produced during senescence by the excited triplet state of chlorophylls are very reactive and oxidize chlorophylls, as well as other electrophilic compounds (Cuny & Romano, 1999).

Photodegradation of chlorophylls usually results in the opening of the tetrapyrrole ring at one of the methine bridges (von Elbe, 2000), leading to the formation of numerous labile intermediates, until finally, the formation of colorless photoproducts (Cuny & Romano, 1999).

The phytyl chain is also highly susceptible to reaction with singlet oxygen, or hydroxyl and peroxy radicals, generated during chlorophyll degradation (Rontani et al., 1995). Photodegradation of chlorophylls is 3 to 4 times more likely to occur in the porphyrin ring than in the isoprenoid phytyl chain (Lee et al., 2014). Singlet oxygen radical attacks the double bonds of the chlorophylls directly, producing hydroperoxides, that are further cleaved to produce radicals (Chen & Huang, 1998).

2.4.6. Other reactions involving chlorophyll

In vitro studies showed that in the presence of phenolic compounds chlorophyll *a* can be degraded by peroxidase, to form 13- hydroxychlorophyll (OHChl) as an intermediate. The presence of this compound in horticultural crops suggests that oxidation, as related to peroxidase, could be involved in postharvest chlorophyll degradation of green fruits and vegetables (Yamauchi, 2015). Whether this mechanism is relevant for food color is not clear.

2.5. Relationship between color and chlorophyll

Chlorophylls are pigments that impart green color to fruits and vegetables. However, the relationship between chlorophyll content and composition and the measured or perceived green color is not always a direct one (Dermesonluoglu et al., 2015). Positive relationships

between these parameters are often reported, but not always. This lack of correlations between color and pigment concentration can be explained by a number of factors. The accuracy of the method for chlorophyll determination (Martins & Silva, 2002) may be at play, but does not explain all instances of lack of correlations.

Total chlorophyll of microwave-dried basil leaves (*Ocimum basilicum*) was closely correlated with chroma values (C*), with a determinant coefficient of R^2=0.93 (Di Cesare et al., 2003). The relation between total color (measured in the L a* b* color space) and chlorophyll content of green chilli (*Capsicum annuum*) puree was described by a linear equation 6 (Ahmed et al., 2000).

$$\left(\frac{La}{b}\right)\Big/\left(\frac{L_0 a_0}{b_0}\right) = k_3\left(\frac{ch}{ch_0}\right) + k_4 \quad (6)$$

where *ch* is the chlorophyll content of green chilli puree at any given temperature (mg L^{-1}), *a* indicates hue on a green (-) to red (+) axis, *b* indicates hue on a blue (-) to yellow (+) axis and *L* indicates lightness. Subscript 0 refers to the respective chlorophyll content of green chilli, and color values at 0 °C reference temperature and k_3 and k_4 are the color and chlorophyll degradation coefficients, per °C (Ahmed et al., 2000).

Significant correlations between chlorophyll content and a* values were reported for soybean varieties harvested at different maturity stages, and dried under two conditions (in oven at 40 °C with circulating air and at ambient temperature around 25 °C). The correlation was higher in seeds dried at ambient temperature than at 40 °C (Sinnecker et al., 2002).

However, in frozen watercress (*Nasturtium officinale*) stored at -7, -15 and -30 ºC none of chlorophyll components (chlorophyll *a*, chlorophyll *b* and total chlorophylls) was significantly correlated with any color parameter (L, a, and b) or their combination (Gonçalves et al., 2009). Frozen green beans (*Phaseolus vulgaris*) stored at the same temperatures also did not show any correlation between total color difference (TDC) and total chlorophyll (Martins & Silva, 2002).

Chlorophyll content it is also not a reliable indicator of green color retention. In some cases, considerable chlorophyll degradation must happen before any visual color difference is observed. Venning et al. (1989) observed that visual color changes were only noticeable after approximately 45% total chlorophyll loss even though instrumental analysis of color detect changes in Hunter L, -a, b values. A 20% loss of chlorophyll caused no, or little change in the color of frozen New Zealand spinach (Jaworska & Kmiecik, 2000). Chlorophyll losses of up to 89% can occur without color changes in frozen unblanched green beans (Philippon et al., 1986; Martins & Silva, 2002).

2.6. Processing effects on chlorophyll and green color losses

2.6.1. Conventional heat treatments

Heat is widely used in food processing to assure enzymatic and microbial stability and eliminate pathogenic food-born microorganisms. Thermal processing method applied to vegetables are generally classified according to the intensity of heat applied as (Aamir et al., 2013; Ahmed et al., 2013):

- Pasteurization, with temperature ranging from 65 to 85 °C;
- Sterilization, using temperatures between 110 and 121 °C or even higher in the ultra-high temperature (UHT) treatment, which can reach 140 to 160 °C;
- Blanching is a short time heat treatment aimed at inactivating enzymes.

Both pasteurization and blanching are considered mild heat treatments aimed at improving food stability. However, these operations have different goals in the food processing diagram. Blanching and pasteurization both inactivate enzymes, but the latter is used reduce the population of spoilage and pathogenic microorganisms (Hui, 2005; Tijskens et al., 2001). Larger logarithmic reductions than those achieved by pasteurization require sterilizations treatments, at higher temperatures, used in canned foods and e.g., in UHT milk.

The effectiveness of any heat treatment depend on two variables: temperature reached and time of exposure. Several factors must be considered to optimize the time-temperature binomial for a thermal treatment, including the type of food, type of microorganism and microbial load, chemical composition of the food material, nutritional value and reaction kinetics for microbial death (Aamir et al., 2013). Maintaining fresh-like quality in an important feature in vegetable processing, but unfortunately even a mild thermal treatment process tends to cause significant changes in quality (Aamir et al., 2013). Given the importance of green color for the overall perception of quality and freshness, a number of studies have investigated the effect of heat on color changes or chlorophyll degradation (Andrés-Bello et al., 2013).

Chlorophyll degradation rate increases with temperature and heating or holding time (Yin et al., 2007). This generalization is supported by observations in several matrices and temperature ranges. Chlorophyll content and green color of green peas decreased with temperature (70 to 100 °C) and heating time (Koca et al., 2007). In broccoli juice chlorophyll degradation to pheophytin occurs at temperatures exceeding 60 °C (Weemaes et al., 1999). The higher accumulation of pheophytin in broccoli juice occurred at 80 °C, decreasing the a*

color coordinate; pheophytin, in turn, decomposes to other degradation products (Weemaes et al., 1999).

Heat treatment of Thompson seedless grapes decrease its chlorophyll content (Zheng et al., 2014), changing color from bright green to olive brown as a consequence of the conversion of chlorophyll (*a* and *b*) to their respective pheophytins, and further degradation to pyropheophytins (Steet & Tong, 1996).

Clearly, the extent of chlorophyll degradation in directly related to the intensity of the thermal treatment, which is characterized by temperature and the duration of heating and holding. High-temperature short-time (HTST) treatments generally provide good results in preserving the green color immediately after processing (Canjura et al., 1991) but chlorophylls are degraded to pheophytin relatively quickly upon subsequent storage or shelf-life (Tan & Francis, 1961).

Transient increases in green color saturation

Even though the reduction of chlorophyll content with heat treatments has been wildly observed, an initial transient increase in green color saturation upon heating has also been reported (Aamir et al., 2014; Tijskens et al., 2001; Lau et al., 2000). The expected decrease of green color due to heat treatment (40 to 96 °C) of broccoli and green beans occurred only after an initial increase in color (Tijskens et al., 2001), and a similar trend was described in green asparagus, during the initial stages of heating between 70 to 98 °C (Lau et al., 2000). Further heating caused the color of the asparagus spears to change from bright green to olive brown, as a consequence of pheophytinization.

This increase in color saturation during tissue blanching is explained by alterations in optical properties associated with the decrease in tissue opacity due to the replacement of air within the tissue with water followed by the release of cellular contents as membranes deteriorate; additionally, it has been hypothesized that the increase in green color saturation is related to the production of visible green components as chlorophyll degrades (Lau et al., 2000; Tijskens et al., 2001).

One of these components are chlorophyllides, which are formed due to the action of chlorophyllase, an enzyme found in green plant tissue (Jones et al., 1963). These were reported as being slightly more stable that chlorophyll (Canjura et al., 1991) and, unlike pheophytins, do not change the chromophore properties and color of their precursor (Turkmen et al., 2006). In fact, chlorophyllides and chlorophylls have similar color (Jones et al., 1963).

The rate of conversion of chlorophyll to chlorophyllides is a function of temperature, due to the activity of the enzyme chlorophyllase which has a maximum activity at 75 °C.

Studies in several vegetable matrices (okra, snap beans, turnip greens, and pickling cucumbers) blanched at 82 °C showed rapid formation of chlorophyllides and other secondary products of chlorophyll degradation a close relationship between the activity of chlorophyllase and the formation of magnesium free derivatives. In contrast, at 100 °C chlorophyllase is inactivated and lower levels of chlorophyllides accumulate (Jones et al., 1963).

Blanching

Frozen vegetables are usually subjected to a moderate heat treatment (blanching) to enhance food safety and inhibit enzyme activity. Enzyme activity during frozen storage can be negligible but reactions can be catalyzed during cooling or after thawing, leading to color and chlorophyll degradation if blanching is not used in the process (Martins & Silva, 2002).

Blanched (2-3 minutes at 100 °C) Brussel retained total chlorophylls content after 8-month storage at -18 °C whereas total chlorophylls decreased in non-blanched samples (Olivera et al., 2008). The chroma of blanched stored Brussel sprouts also decreased less on blanched samples during 8 month storage, compared to non-blanched samples (Olivera et al., 2008). Blanched (2 min at 100 °C) frozen beans preserved the initial chlorophyll content and color during 250 days of storage at -7, -15 and -30 °C (Martins & Silva, 2002). At storage temperatures of -7 and -15 °C color losses occurred at significant fast rates, with great color differences after 15 and 30 days of storage, respectively. Green been chlorophyll stability was lower than color at all storage temperatures, a fact that was attributed to different quantification methods for color measurement and chlorophyll mass measurement(Martins & Silva, 2002).

2.6.2. Low temperature methods

Since chlorophyll degradation reaction rates increase with increasing temperature as described by the Arrhenius equation, low temperature reduces chlorophyll degradation rates. Preservation of food at low temperature includes two distinct temperature ranges: refrigeration and freezing. Refrigeration is the application of temperatures above the freezing point of food, while freezing is usually under −18 °C (Hui & Evranuz, 2016). Freezing is more effective at preserving the chlorophyll content of vegetable matrices because of the additive effect of the lower temperature range and change of water phase from liquid to solid (Hui & Evranuz, 2016).

Changes in chlorophyll lead to modification in the color of green vegetables with freezing and frozen storage. These changes can be due to chemical (removal of Mg^{2+} ion from the porphyrin ring) and enzymatic pathways, mainly chlorophyllase effect on chlorophylls, resulting in chlorophyllides and pheophytins, although other enzymes can be involved (Gonçalves et al., 2009).

Due to chlorophylls sensitivity to temperature it is possible to stabilize green color using frozen storage. The color value -a is highly sensitive to storage temperatures and temperature fluctuations, while the b-coordinate, although exhibiting faster degradation rate than -a, has lower sensitivity to storage temperature (Martins & Silva, 2002).

Lisiewska & Kmiecik (1997) studied frozen parsley of two cultivars stored for 9 months at -20 and -30 °C. Overall chlorophyll retention at -20 °C was 78-95% and higher retention was observed in the cultivar with higher initial chlorophyll content. During freezing and storage at -30 °C no significant differences in chlorophyll content were observed, which rendered previous blanching unnecessary.

Frozen kiwifruit pulp stored at several temperatures revealed that storage temperature has a great effect on total chlorophyll and color of frozen kiwifruit (Venning et al., 1989). Rapid chlorophyll degradation and color loss were observed at -9 ºC. At or below -18 ºC (commercial frozen storage) color remained stable for 12 months. Chlorophyll stabilization can be achieved in frozen foods at temperatures below -18 or -20 ºC (Venning et al., 1989; Lisiewska & Kmiecik, 1997).

2.7. Emerging thermal treatments and their effects on color and chlorophyll

2.7.1. Ohmic heating

In ohmic heating, heat is generated within the food product by the resistance offered by the food matrix to electric current (Yildiz et. al, 2010). This heating process is faster and more uniform than traditional heat exchange systems (Singh & Heldman, 2013). Ohmic heating can be applied in several processes, including blanching, evaporation, dehydration and pasteurization (Krokida et. al, 2001).

Yildiz et al. (2010) heated spinach puree from 30 ºC to various temperatures (60 ºC, 70 ºC, 80 ºC or 90 ºC) by ohmic and conventional heating. Four different voltage gradients were applied, in the range of 10–40 V cm^{-1} in the ohmic treatment, while conventional heating was conducted at constant temperature in a water bath. Puree heated faster in the ohmic system than in the heat exchanger. However, there was no effect of voltage on chlorophyll content, and it was concluded that chlorophyll levels of spinach puree were maintained in this voltage range at 70 °C during holding periods of 0-10 min.

Artichoke heads blanched by conventional methods with hot water (100 ºC) and ohmic (24 V cm^{-1}, 80 ºC) blanching treatments showed an increase in lightness (L* values) with blanching time in the ohmic blanched heads but a L* decreased with blanching time in conventionally blanched heads. Overall the artichokes blanched by ohmic treatment retained

the bright green color typical of the raw product, while the color of samples treated with the conventional method changed from their initial bright green color to a brownish olive green (Guida et. al, 2013).

2.7.2. Microwave heating

Microwaves are electromagnetic radiation with frequencies ranging from 300 MHz to 300 GHz. Heating by microwave radiation can be used in food processing for drying, pasteurization and sterilization (Chandrasekaran et. al, 2013). Microwave heating is volumetric, and has higher penetration power, faster heating rates, higher thermal efficiency and shorter processing times, and induces smaller changes in flavor and nutritional composition than conventional heating processes (Huang et. al, 2007).

When compared to conventional heating methods, microwave heating had a better effect on quality of kiwi fruit puree and green tea. Microwaved kiwi fruit puree (1000 W-340 s) had smaller color changes when compared to conventionally heating (97 ºC – 30 s) (Benlloch-Tinoco et. al, 2015), while chlorophyll content of tea leaves heated by microwave was higher and more stable during storage than that of oven-heated samples, indicating that microwave-heating can reduce chlorophyll decomposition (Huang et al., 2007).

Color and chlorophyll content in of herbs such as marjoram (*Marjona hortensis*) and rosemary (*Rosmarinus officinalis*) were better preserved after, microwave blanching than after water and steam blanching (Chandrasekaran et al., 2013). Overall, microwaving preserves colors better than other convention thermal techniques (Benlloch-Tinoco et al., 2015).

2.7.3. Thermosonication

Thermosonication treatments combine ultrasound with heat (Chemat et. al, 2011). Thermosonication can be used as an alternative to hot-water blanching, minimizing color changes, since blanching times can be reduced (Rodrigues & Fernandes, 2012). A treatment with ultrasonic intensity of 11.94 W cm^{-2} at 85 ºC for 60 s allowed the retention of 96.7% of total chlorophyll in argy wormwood (*Artemisia argyi*) leaves, a better retention than that of conventional blanching, mainly due to shorter blanching times of thermosonication (Xin et al., 2015).

2.7.4. Non-thermal treatments

High-pressure processing

High-pressure processing (HPP) applies isostatic pressure to food in the range of 100 to 1000 MPa at temperatures of -20 to 60 °C, in order to inactivate microorganisms and some enzymes (Oey et. al, 2008). This technique extends shelf life with minor changes in sensorial and nutritional properties of foods (Rodrigues & Fernandes, 2012). At low and moderate temperatures, HPP treatments have a negligible effect on chlorophyll and other pigments. However, due to incomplete inactivation of enzymes and microorganisms undesired chemical reactions may occur, resulting in changes in color during storage (Oey et. al, 2008). This method is less detrimental to low molecular weight food compounds such as flavoring agents, pigments, and vitamin, as covalent bonds are not affected by pressure (Wang et al., 2013).

Pressure affects the green color via inactivation of enzymes or influence in membrane permeability, as indicated by the study of Chen et al. (2015) with asparagus juice at various pressure values (200, 400 and 600 MPa) and treatment times (10 and 20 min). The color of the juice treated at 400 and 600 MPa was better preserved than that of the juice treated at 200 MPa. The authors assumed that this was due to the increase of permeability of chloroplast membranes due to high pressures, with consequent leakage of chlorophyll into the intercellular space (Chen et al., 2015).

HPP treatments (400 MPa for 4min and 500 MPa for 2 min) did not affect the levels of chlorophyll *a* and *b* in cucumber juice in relation to control samples, in contrast with the decreases induced by thermal pasteurization (85 ºC for 15 s) (Zhao et al., 2013). Samples treated with HPP also had better chlorophyll stability compared to pasteurized samples. HPP processing delay green color deterioration of spinach puree (Wang et al., 2012). Overall results obtained in studies conducted by this author in spinach puree were in accordance with results by Zhao et al. (2013). Contents of chlorophyll *a* in HPP treated samples were significantly higher than those treated with thermal methods for all studied storage periods, with degradation rate constants for HPP treated samples about 40% to 60% as low as that of thermal treated samples.

Pulsed Electric Fields

Pulsed electric field (PEF) technology applies electric pulses to a product placed in a treatment chamber confined between electrodes (Soliva-Fortuny et. al, 2009). These

treatments can be of moderate to high intensity (generally 20–80 kV cm^{-1}) (Hui & Evranuz, 2016). The application of PEF to food aims at microbial and enzyme inactivation (Rodrigues & Fernandes, 2012), with minimal impact on the food item (Hui & Evranuz, 2016). Although this technology is not yet extensively used in commercial food processing, PEF may become an alternative to thermal pasteurization of liquid foods (Soliva-Fortuny et al., 2009).

The effect PEF on chlorophylls is virtually unknown. One study found that increasing field strength (up to 60 kV cm^{-1}) increased the green color of spinach puree, due to destruction of microorganisms and enzymes responsible for chlorophyll degradation mechanisms (Yin et. al, 2007). However, at fields higher than 60 kV/cm there appeared to be a detrimental effect on color of spinach puree samples, although the reason for this phenomena is not clear (Yin et. al, 2007).

Ionizing radiation

The forms of ionizing energy (irradiation) that can be used in food processing are gamma rays (γ), X-rays and electron beans (β particles) (Odueke et al., 2016). Ionizing radiation is defined as a radiation with enough energy to remove tightly bound electrons from atoms, creating ions, and has been mainly used to eliminate potentially harmful organisms from food (Rodrigues & Fernandes, 2012), but it is also used to inhibit ripening and senescence, post-harvest growth of mushrooms and asparagus, spouting of tuber, bulb and root vegetables, as well as control of post-harvest disease (Kader, 1986). Among these treatments, gamma radiation is the most widely used.

Gamma irradiation insures microbiological safety of perishable foods (Kamat et. al, 2003), and is effective in reducing mold and bacterial contamination and delaying ripening of climacteric fruits (Wani et. al, 2008).

Gamma rays originate in the nucleus of a radioactive atom (Rodrigues & Fernandes, 2012). The most commonly used radioactive nucleotides in food irradiation are cobalt-60 and cesium-137 (Kovács & Keresztes, 2002).

Chlorophylls are sensitive to irradiation. The rate of decrease in chlorophyll content of green beans and broccoli is directly related to the absorbed dose in the range 4.9 to 92.9 kGy. Vegetable samples irradiated with 5 kGy maintained chlorophyll levels during 3 month storage (Fan & Sokorai, 2007). Chlorophyll content of alfalfa sprouts irradiated at 2.6 kGy had lower chlorophyll a to b ratio compared to non-irradiated ones, while total chlorophyll was slightly promoted by irradiation. However, irradiation did not have a consistent effect on total chlorophyll of the sprouts (Fan et. al, 2003).

The decrease of chlorophyll due to irradiation was also demonstrated by Ling et al. (2008). Irradiated *Citrus sinensis* plantlets had lower chlorophyll content than non-irradiated

plantlets, although the intensity of the irradiation (10, 20 or 30 Gy) did not affect chlorophyll content of irradiated samples. However, 50 Gy induced 76.9% chlorophyll reduction compared to non-irradiated plantlets. This leads to the conclusion that at lower doses of gamma irradiation, chlorophyll is virtually unaffected. Chlorophyll *a* was relatively higher than chlorophyll *b* in all samples, although irradiation resulted in higher degradation of chlorophyll *a* as opposed to chlorophyll *b*.

Low dose gamma irradiation (1 kGy) are effective bactericide treatment for fresh coriander leaves, improving quality of storage leaves up to 2 weeks at 8-10 ºC, while maintaining total chlorophyll content (Kamat et al., 2003).

Shelf life of irradiated pears was also improved by irradiation (0.8-2.0 kGy), while resulting in higher chlorophyll contents throughout storage when compared to un-irradiated samples. Highest chlorophyll values were observed at 1.5-1.7 kGy. This may be attributed to an inhibitory effect of irradiation on the activity of chlorophyllase enzyme, as well as possible stress signals triggered due to free radicals produced during irradiation, resulting in slower degradation of chlorophyll (Wani et al., 2008).

Ultraviolet

Ultraviolet (UV) radiation treatment is a non-thermal method of preservation used for water and air treatments and nonfood and food contact surface disinfection (Rodrigues & Fernandes, 2012).

UV radiation can be subdivided into three ranges of the electromagnetic spectrum: longwave UV-A radiation (320-400 nm), medium-wave UV-B radiation (280-320 nm), and short-wave UV-C radiation (200-280 nm) (Urban et al., 2016).

UV-C treated (0.03 kJ m^{-2}) bananas peels (Musa AAA group, Cavendish subgroup, cv. Cavendish), stored at 8 and 25 °C significantly maintained higher chlorophyll levels when compared to control sample. This significant difference was attributed to the inhibition of chlorophyllase and chlorophyll-degrading peroxidase activities, due to UV-C action (Pongprasert et al., 2011). UV-C treated (10 kJ m^{-2}) broccoli (Brassica oleracea L.) florets maintained higher chlorophyll levels and hue during 5 d storage compared to control sample (Bücherta et al., 2011). Costa et al. (2006) obtained similar results, also in broccoli florets (cv. de Cicco). The florets were treated with of UV-C light at 4, 7, 10 and 14 kJ m^{-2}, observing the highest chlorophyll retention in samples treated with 10 kJ m^{-2}. Overall chlorophyll *a* and *b* degradation was delayed, as well as yellowing and pheophytins increase during storage, due to inhibition of chlorophyllase and chlorophyll peroxidase. Although an initial increase in Mg-dequelatase was observed after treatment, after 4 and 6 days the activity decreased to values lower than in control samples (Costa et al., 2006).

UV-B radiation applied to yellowing broccoli florets during storage at 15 ºC showed higher retention of color after irradiation when compared to UV-A irradiation, even though both doses of UV were similar (Aiamla-or et. al, 2010). These authors also investigated the effect of UV-B irradiation on chlorophyll content of stored broccoli florets, concluding that UV-B doses of at least 8.8 kJ m^{-2} were effective on delaying the decrease of chlorophyll *a* and *b* content, as well as hue. These values of irradiation showed a suppression of chlorophyllase and chlorophyll-degrading peroxidase activities, as well as Mg-dechelatase activity.

This positive effect on chlorophyll content due to UV-B irradiation was also observed by Srilaong et. al (2011). Limes (*Citrus latifolia* Tan.) were treated with 8.8 kJ m^{-2} and 13.2 kJ m^{-2} and stored at 25 ºC in darkness. While UV-B treatments at 8.8 kJ m^{-2} showed to delay chlorophyll degradation, while also maintaining the external qualities of the fruit, at UV-B irradiation of 13.2 kJ m^{-2} there was an increase of water loss and enhancement of yellowing of the fruit. Chlorophyll content of the lime peel treated with UV-B was maintained at approximately 12.5–14 mg 100 g FW^{-1}, control sample suffered a sharp decrease after the 15th d of storage.

UV radiation treatments can also be used pre-harvest to increase chlorophyll content of certain cultivars, as shown in studies by Caldwell & Britz (2006) in greenhouse grown leaf lettuce (*Lactuca sativa*) cultivars. In this study, green leaf and red leaf lettuce varieties were grown in a greenhouse under control, receiving supplemental UV-A and combined UV-A and UV-B radiation for 9 days, before harvest and analysis. In general, pre-harvest application of UV radiation showed to increase chlorophyll *a* and *b* content, for all green leaf lettuce samples, while reducing the levels of these compounds in red leaf lettuce. Combined UV-A and UV-B treatments were the most effective in increasing chlorophyll (*a* and *b*) content in green lettuce.

Chapter 3

Materials and Methods

This chapter describes the materials and methods used to set up and conduct the experiments.

3.1. Plant material

Leaves of spinach (*Spinacia oleracea* 'Manhattan'), parsley (*Petroselinum crispum*, plain leaf cultivar), broccoli (*Brassica oleracea* 'Parthenon') and lettuce (*Lactuca sativa*) were freshly harvested in Loures, Portugal, in the period between February and March of 2016, and were processed within 5 hours after harvest.

Lettuce seedlings were grown in styrofoam trays and used when the plant had 6.9 ± 1.0 leaves, 13.7 ± 0.8 cm high.

Leaves were washed with tap water, and sorted to remove yellow leaves, and petioles in spinach and parsley. Only outer and greener sides were used in the case of broccoli leaves and lettuce, with removal of petiole and the midrib.

3.2. Homogenate preparation

A liquid matrix was prepared from the raw material and subjected to several treatments to evaluate the effect of (i) plant material (freshly harvested), (ii) pasteurization (90 °C for 60 s) (iii) food additives (sodium bicarbonate and zinc chloride) and pH adjustment, (iv) foliar zinc treatments and (v) shelf-life (16 d at 20 °C).

After sorting, the leaves were manually cut with a sharp knife into smaller pieces for subsequent homogenization. Leaf pieces (100 g) were homogenized in distilled water (900 mL) to make up a 10% liquid suspension. Homogenization was performed in two steps: with a manual blender for 3 min. to assure size reduction followed by an homogenization of the resulting mixture for 3 min. at 10 000 rpm (T25 Basic, IKA Labortechnik, Janke & Kunkel, Stanfen, Germany) in 30 second runs, until uniform consistency.

After homogenization the liquid matrix was transferred to 170 mL glass bottles and capped. After bottling the samples were pasteurized for 60 s at 90 °C in a water bath (model 2971, Thermo Fisher Scientific, Waltham, MA, USA) with reciprocal shaking at 50 rpm. Sample temperature was monitored using a digital thermometer. After the heat treatment the samples were cooled in a water bath and maintained at 20 °C in the dark.

3.3. Food additives and pH treatments

Both additives were added to the leaves prior to homogenization. Sodium bicarbonate ($NaHCO_3$) was added at 5 mg L^{-1}, and zinc chloride ($ZnCl_2$) was used at 100 mg L^{-1}. Following the production and pasteurization of the homogenate (vide 3.1.4), pH of the suspension was

adjusted to 4.5, 6.0 and 8.5. Sodium hydroxide (NaOH) was used to increase pH and diluted sulfuric acid (H_2SO_4) to lower the pH.

3.4. Pre-harvest foliar application of zinc

Lettuce plants were sprayed with a $ZnCl_2$ solution at 200 mg L^{-1} (Zn^{2+} at 100 mg L^{-1}) wetting the leaves until dripping. The plants were sprayed twice, 3 and 1 days before harvest. Control samples (CT) were sprayed with deionized water. After harvest, lettuce leaves were processed as described (section 3.2). The effect of combined foliar treatments and additives on color maintenance was evaluated by the production of a lettuce homogenate with a zinc chloride solution (100 mg L^{-1}) to make up a 10% lettuce homogenate. Figure 7 summarizes the work flow of sample preparation with pre-harvest zinc application.

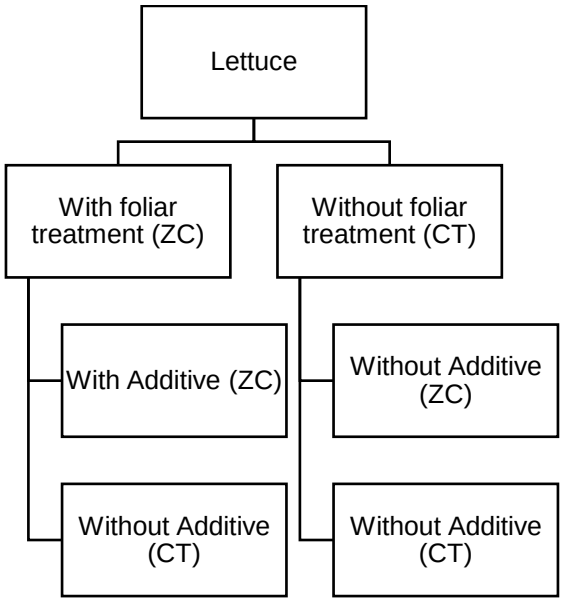


Figure 7 - Experimental setup for foliar application trials.

3.5. Shelf-life studies

The pasteurized homogenates were immediately analysed or maintained in controlled temperature room at 20 °C in the dark for shelf-life studies. These samples were analysed at 2 to 3 days intervals for up to 16 days.

3.6. Analytical procedures

3.6.1. pH

pH was measured by potenciometry with a pH meter (PH-2005 pH meter, JP Selecta, Barcelona, Spain) compensated for temperature. One measurement was made in each of 3 independent replicate bottles per treatment.

3.6.2. Soluble solid content

The soluble solid content (SSC) was measured using a digital refractometer (HI 96801, Hanna Instruments, Woonsocket, USA) at room temperature. One measurement was made in each of 3 independent replicate bottles per treatment.

3.6.3. Color parameters

Color was measured under standard conditions as follows. Liquid (6 mL) was transferred to a clear cylindrical (d=35 mm; h=10 mm) polypropylene container and capped with a polypropylene lid. The cylinder containing the liquid sample was placed on top of the colorimeter window (8 mm) and completely covered with an opaque container to prevent transmission of external light to the sensor.

Color was measured in the CIE L*a*b* space with a tristimulus colorimeter (CR 400, Konica-Minolta, Tokyo, Japan) with the C illuminant. The instrument was calibrated using a standard white tile (L*=97.10, a*=0.19, b*=1.95) before each set of measurements.

A total of 3 measurements were made per sample replicate and the L*, a*, and b* data converted to chroma (C*) and hue angle (h°) using equations 7 and 8, respectively.

$$C^* = \sqrt{(a^{*2} + b^{*2})} \tag{7}$$

$$^\circ h = arctg\left(\frac{b^*}{a^*}\right) \tag{8}$$

3.6.4. Photographic record

The samples placed in the clear polypropylene cylinders as described above were photographed with a digital camera (EOS 550 D, Canon, Tokyo, Japan) equipped with Canon EFS 18-135 mm lens. The camera was set to ISO 200, shutter speed 1/6, aperture F5.6.

Camera was positioned at a distance of 40 cm from samples, in a photo studio, with artificial lighting from both sides, and no other external light sources.

3.6.5. Chlorophyll and carotenoid content

Aliquots (10 mL) of the homogenate were diluted in 40 mL of acetone and the resulting mixture incubated for 30 min in a reciprocal shaking bath (model 2971, Thermo Fisher Scientific, Waltham, MA, USA) in the dark. The samples were then filtered through a cellulose filter paper (73 g, Deltalab S.L., Barcelona, Spain) and the filtrate centrifuged (Universal 16 R, Hettich Zentrifugen, Tuttingen, Germany) at 2600 x *g*, under refrigeration (4˚C), for 15 minutes. The supernatant was collected and used for spectrophotometric readings. Extraction was performed in each of the 3 independent sample replicates.

Absorbance was measured at 440.5 nm, 663.6 nm and 646.6 nm with a CE 1011 spectrophotometer (Cecil Instruments, Cambridge, England). The concentration of chlorophyll *a*, chlorophyll *b*, total chlorophylls, and total carotenoids was calculated from the absorbance measurements using the extinction coefficients and equations 9 to 12 (Yang et al., 1998).

$$Chl\ a = 12.25A_{663.6} - 2.55A_{646.6}\ (\mu g\ mL^{-1}) \quad (9)$$

$$Chl\ b = 20.31A_{646.6} - 4.91A_{663.6}\ (\mu g\ mL^{-1}) \quad (10)$$

$$Chl\ a+b = 17.76A_{646.6} + 7.34A_{663.6}\ (\mu g\ mL^{-1}) \quad (11)$$

$$Car = 4.69A_{440.5} - 0.267Chl\ a+b\ (\mu g\ mL^{-1}) \quad (12)$$

3.7. Statistical analysis

Data were subjected to three-way factorial analysis of variance (ANOVA) followed by the Fisher least significant difference (LSD) test for mean separation. Statistical analyses were performed with Statistica™ Software v.7.0 (StatSoft, Inc., 2004).

The relationship between color (hue angle) and chlorophyll concentration was analysed by linear regression after exploratory graphical analysis.

Chapter 4

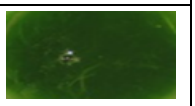
Results and Discussion

This chapter contains the results obtained in the experimental trials conducted on vegetable homogenates and their discussion. The results are presented in 7 sections. The initial caracteristics of the vegetable leaf homogenates are presented in section 4.1, while the effect of heat treatment on these caracteristics is presented in section 4.2. The effect of food additives and pH on leaf homogenates before and after heat treatment is discussed in section 4.3 and 4.4, respectively. Section 4.5 and 4.6 comprise the results of foliar zinc application on characteristics of leaf homogenates before and after heat treatment, respectively. In section 4.7 the correlation between chlorophyll content and hue value for the vegetable homogenates in study is discussed.

4.1. Initial characterization of vegetable leaf homogenates

Immediately after homogenization the raw materials had the characteristics summarized in Table 3. Leaf homogenates had pH values ranging from 6.0 to 6.4 (Table 3). These values fall within the pH range of green vegetables, between 5 and 7 (Aamir et al., 2013). pH values reported elsewhere are 6 to 7 for spinach and 5.2 to 6.3 for broccoli florets (Aamir et al., 2013).

Table 3 – pH, soluble solid content, color parameters and photographic record of vegetable homogenate samples produced from freshly harvested plant material. Values are means ± s.d. (*n*=3). Means followed by a different letter are significantly different according to Fisher's LSD test at α=0.05 (within columns).

Plant Material	pH	SSC	Color			
			L*	C*	hue angle	Photo record
Spinach	6.2^{d}±0.1	0.5^{b}±0.1	26.0^{b}±0.1	7.3^{b}±0.3	129.3^{b}±1.0	
Parsley	6.4^{a}±0.1	0.8^{a}±0.1	26.0^{b}±0.1	7.5^{b}±0.4	132.7^{b}±0.8	
Broccoli	6.1^{b}±0.1	0.8^{a}±0.1	27.2^{a}±0.2	9.4^{a}±0.3	132.8^{a}±0.1	
Lettuce	6.0^{c}±0.1	0.5^{b}±0.1	25.3^{c}±0.1	4.5^{c}±0.1	109.8^{c}±3.2	

Soluble solid content was low, as expected in a homogenate containing 10% of vegetable leaves (Table 3). Soluble solid content was slightly higher (0.8%) in parsley and broccoli samples than in spinach and lettuce (0.5%).

Hue of parsley and broccoli samples was of 132.8°, higher (greener hue) than the 129.0° and 109.8° in spinach and lettuce samples, respectively. Hue angle does not completely summarize the visually perceived colors (Table 3), but it is good indication of green color. Hue alone is not sufficient to describe color in a way that is consistent with human eye perception. However, differences in L* and C* values can account for the difference in the perceived color of samples. Broccoli samples appear darker than spinach, parsley and lettuce samples, and had the highest L* (27.2) and C* (9.4) values. Spinach and parsley samples had the same L* value (26.0), similar C* (7.3 in spinach and 7.5 in parsley samples) and hue (129.3° in spinach and 132.7° in parsley), all of which point to samples having the same color. The difference in hue and saturation of lettuce samples is apparent by the L*, C* and hue values (25.3, 4.5 and 109°, respectively) lower than those of spinach, parsley and broccoli. These values lead to a

darker, less saturated and less green color, as seen by the visually perceived color (Table 3). Visually perceived color of lettuce samples highlights the importance of the choice of raw material, since the original bright green color of lettuce leaves turned into an olive green color immediately after processing.

Chlorophyll *a* and chlorophyll *b* content of the homogenates prepared from different raw materials is shown in Figure 8.

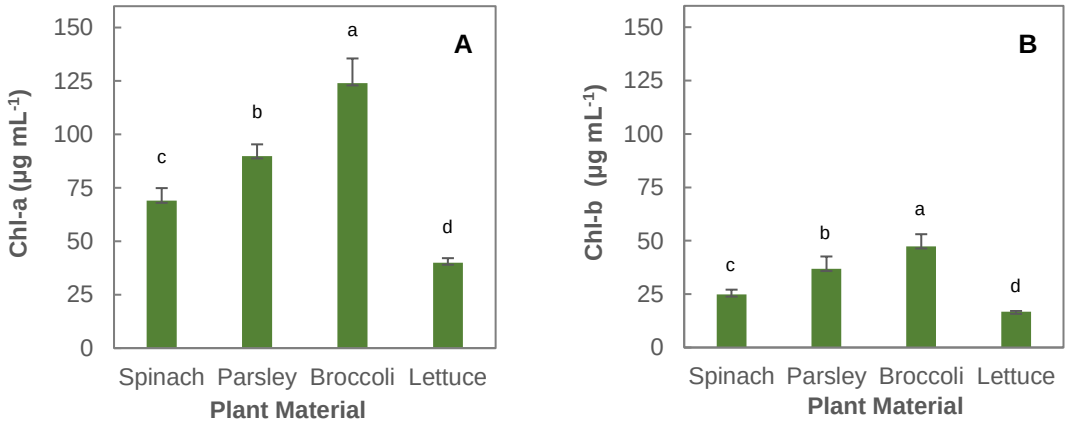


Figure 8 - Plant material influence on chlorophyll *a* (A) and chlorophyll *b* (B) content of vegetable homogenates. Error bars are standard deviation (*n*=3). Means followed by a different letter are significantly different according to Fisher's LSD test at α=0.05.

Lettuce homogenates had the lowest chlorophyll *a* content (40.0 µg mL^{-1}), followed by those of spinach (69.0 µg mL^{-1}), parsley (89.8 µg mL^{-1}) and broccoli (124.0 µg mL^{-1}) samples. The concentration of chlorophyll *b* was lower than that of chlorophyll *a* but the relative content of the different matrices was in the same order: lowest in lettuce homogenates (16.8 µg mL^{-1}), followed by those prepared from spinach (24.8 µg mL^{-1}), parsley (36.8 µg mL^{-1}), and broccoli with the highest content(47.4 µg mL^{-1}) (Figure 8).

Chlorophyll content, expressed on a fresh mass basis, has been reported for parsley as 890 mg kg^{-1} and 288 mg kg^{-1} for chlorophyll *a* and chlorophyll *b,* respectively (Belitz et al. (2009). Spinach contains 946 mg kg^{-1} of chlorophyll *a* and 202 mg kg^{-1} of chlorophyll *b* (Belitz et al., 2009). Green lettuce leaves have a chlorophyll *a* concentration ranging from 4.6 to 33.5 µg mg^{-1} and 3.2 to 25.1 µg mg^{-1} of chlorophyll *b* (Cadwell & Britz, 2006). The concentrations measured in this study are higher than those reported in the literature for spinach, parsley and broccoli. In the case of lettuce samples, determined chlorophyll *a* values are close to those mentioned by Cadwell and Britz (2006) and chlorophyll *b* content is within the range of values determined by these authors.

Total chlorophylls content of spinach, parsley, broccoli and lettuce homogenates is presented in Figure 9.

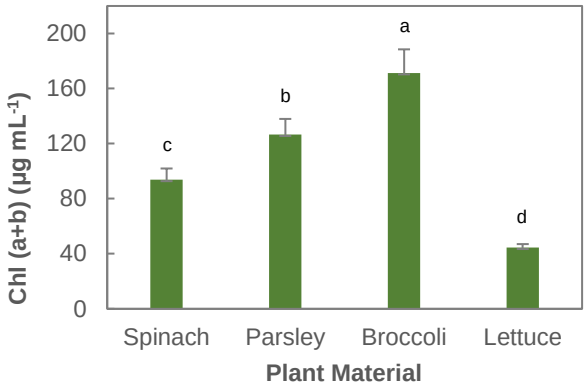


Figure 9 - Plant material influence on total chlorophylls content of vegetable homogenates. Error bars are standard deviation (*n*=3). Means followed by a different letter are significantly different according to Fisher's LSD test at α=0.05.

Total chlorophyll varied 3-fold in the homogenates prepared from different raw materials. Higher in broccoli samples (171.4 µg mL^{-1}), followed by parsley (126.6 µg mL^{-1}) and spinach (93.7 µg mL^{-1}) samples. Lettuce samples had the lowest total chlorophyll content of all samples, with 44.4 µg mL^{-1} (Figure 9). Higher concentrations of chlorophyll can lead to darker or more intense green color of produced sample mixture, as seen in the case of broccoli (Table 3). Although spinach homogenates had lower chlorophyll content than broccoli, no visually perceived color differences were detected for these samples. In the case of lettuce samples, lower total chlorophyll content was consistent with the visually perceived color, which was yellow-green (Table 3).

The ratios between chlorophylls *a* and *b* in the four vegetable homogenates are shown in Figure 10. Overall the ratio between chlorophyll *a* and chlorophyll *b* ranged from 2.4 (lettuce) to 2.8 (spinach) (Figure 10). Chlorophyll *a* and chlorophyll *b* generally occur in plants with ratio of 3:1 (Gaur et al., 2006), chlorophyll *a* occuring at higher concentrations than chlorophyll *b*.

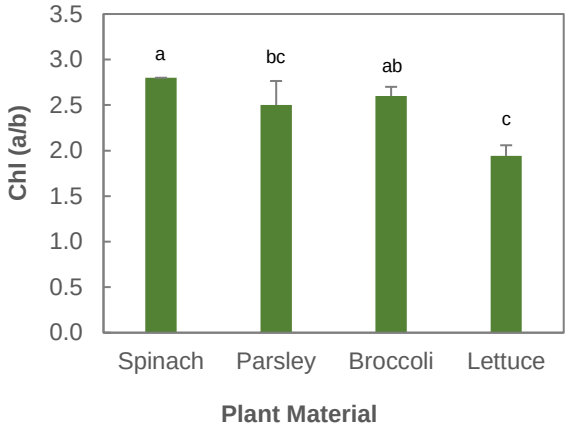


Figure 10 - Plant material influence on the ratio between chlorophyll *a* and chlorophyll *b* of vegetable homogenates. Error bars are standard deviation (*n*=3). Means followed by a different letter are significantly different according to Fisher's LSD test at α=0.05.

Carotenoid content of spinach, parsley, broccoli and lettuce homogenates is presented in Figure 11.

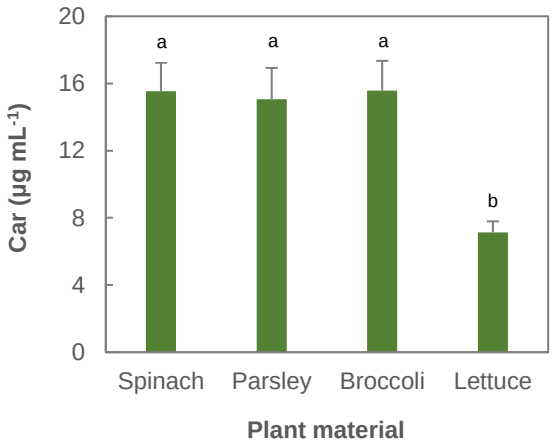


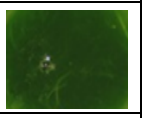
Figure 11 - Plant material influence on carotenoid content of vegetable homogenates. Error bars are standard deviation (*n*=3). Means followed by a different letter are significantly different according to Fisher's LSD test at α=0.05.

All homogenates had similar carotenoid content (15 µg mL^{-1}), except those prepared from lettuce, where lower carotenoid content was determined (7.1 µg mL^{-1}). However, the method used for carotenoid determination did not allow for an accurate analysis of this parameter, and led to higher incertitude to the veracity of determined values.

4.2. Effect of heat treatment on quality parameters of homogenates

The variables pH, SSC, and color were measured immediately before and after pasteurization at 90 °C for 60 s. The differences are shown in Table 4.

Table 4 - pH value, soluble solid content, color and photographic record of vegetable homogenate produced from freshly harvested plant material after heat treatment. NHT- No heat treatment; HT- Heat treatment. Values are means ± s.d. (*n*=3). Means followed by a different letter are significantly different according to Fisher's LSD test at α=0.05 (within columns).

Plant Material	Heat treatment	pH	SSC	Color			
				L*	C*	hue angle	Photo record
Spinach	NHT	6.2^{c}±0.1	0.5^{b}±0.1	26.0^{d}±0.1	7.3^{f}±0.3	129.3^{b}±1.0	
	HT	6.3^{b}±0.1	0.5^{b}±0.2	25.1^{e}±0.1	7.3^{f}±0.2	125.3^{d}±0.5	
Parsley	NHT	6.4^{a}±0.1	0.8^{a}±0.1	26.0^{d}±0.1	7.5^{f}±0.4	132.7^{a}±0.8	
	HT	6.0^{g}±0.1	0.2^{c}±0.1	33.2^{b}±0.2	16.3^{b}±0.1	125.7^{d}±0.5	
Broccoli	NHT	6.1^{d}±0.1	0.8^{a}±0.1	27.2^{c}±0.2	9.4^{c}±0.3	132.8^{d}±0.1	
	HT	5.6^{f}±0.1	0.7^{a}±0.1	33.8^{a}±0.1	16.8^{a}±0.2	126.4^{d}±0.2	
Lettuce	NHT	6.0^{g}±0.1	0.5^{b}±0.1	25.3^{e}±0.1	4.5^{e}±0.1	109.8^{c}±3.2	
	HT	5.8^{e}±0.1	0.5^{b}±0.1	27.3^{c}±0.1	6.1^{d}±0.2	109.8^{c}±3.2	

pH was not significantly affected by pasteurization. A sharp decrease in the SSC, from 0.8% to 0.2%, was observed after pasteurization in parsley homogenate but little or no change was observed in the SSC of spinach, broccoli and lettuce samples.

Color changed during pasteurization of homogenates prepared from all raw materials except lettuce. These changes were evident in the visually perceived color, with decrease of intensity of initial green color and color shift, from bright green to a yellower green tone. This

color shift may be attributed to the formation of pheophytins, which have a characteristic olive green color, due to chlorophyll conversion led on by heat application. Although the initial color of lettuce samples was already olive green, it was possible to observe a color shift to a browner tone, also possibly due to pheophytin presence (Steet & Tong, 1996).

The changes is perceived color are partially substantiated by objective measurements. Hue angle decreased after pasteurization in all samples, reflecting the color shift toward yellow hues. from 132.7° to 125.7° in parsley samples, from 132.8° to 126.4° in broccoli samples and from 129.3° to 125.3° in spinach samples. An overall increase in C* value with heat treatment was observed in all samples except in spinach, where C* value remained constant (7.3). This fact can account to the less saturated green color of these samples, when compared to parsley and broccoli homogenates. Broccoli and parsley samples had an increase in color saturation (C*), respectively, from 9.4 to 16.8 and from 7.5 to 16.3 indicating increased hue saturation. Lightness (L*) increased from 26.0 to 33.2 in parsley samples and from 27.2 to 33.8 in those of broccoli. These increases in lightness are apparent in the photographic record of Table 4. Although there were no differences in the hue of lettuce homogenates, C* and L* increased slightly in this matrix.

Figure 12 shows the immediate effect of heat treatment on homogenate samples, on chlorophyll a*,* chlorophyll b*,* as well as total chlorophylls of vegetable homogenates.

Chlorophyll content decreased after heat treatment (Figure 12 A, B e C), consistent with the temperature effect on chlorophyll stability and chlorophyll *a* and chlorophyll *b* conversion to the respective pheophytins (Steet & Tong, 1996). While loss of chlorophyll *a* was similar in spinach, parsley and broccoli samples (~22%), the reduction in lettuce was percentually higher (~27%). Chlorophyll *b* degradation was more dependent of raw material: chlorophyll *b* loss in spinach samples was higher, with 46%, followed by parsley samples, with 38% loss, broccoli leaves samples, with 12% loss and lettuce samples, with only 9% loss.

Loss of total chlorophylls was higher in spinach samples (28%), followed by parsley samples (25%), lettuce samples (22%) and broccoli samples (19%). This result may point to higher chlorophyll stability in broccoli samples due to lower chlorophyll-pheophytin conversion rates, as opposed to spinach samples, where chlorophyll content decreased the most with heat application. Other factors, intrinsic to the choice of raw material, may be responsible for chlorophyll maintenance and its protection against external heat application, although the nature of this effect was not explored.

The chlorophyll degradation ranking is consistent with the hue values for spinach, parsley and broccoli samples, in which spinach samples had the lowest hue (125.3°) and broccoli samples had the highest hue (126.4°). The same cannot be said about lettuce samples that, as previously mentioned, maintained the hue angle.

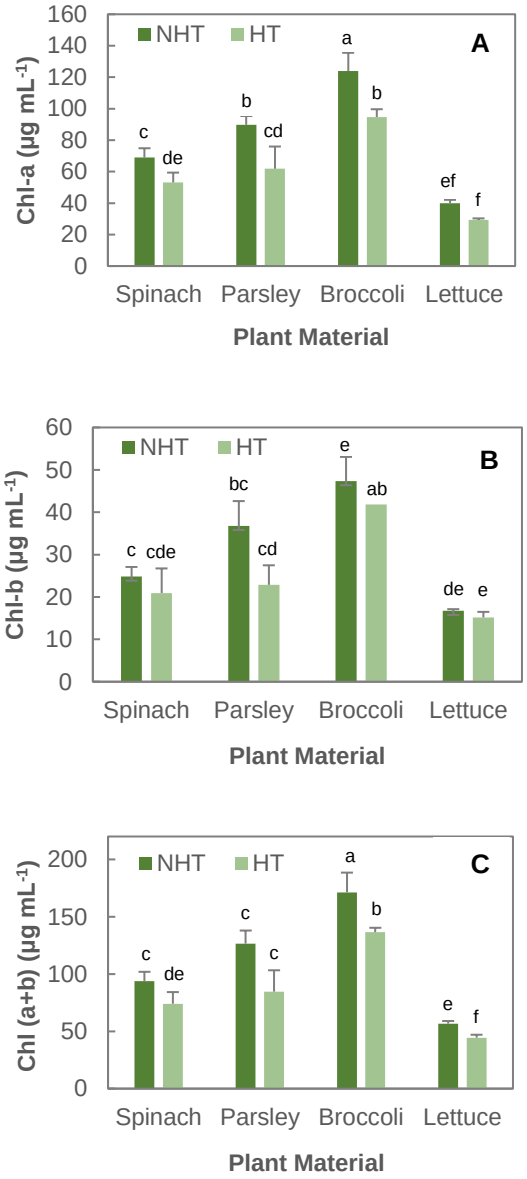


Figure 12 - Heat treatment effect on chlorophyll *a* (A), chlorophyll *b* (B), total chlorophyll content (C) on vegetable homogenates from different plant materials. NHT – No heat treatment; HT- heat treatment. Error bars are standard deviation (*n*=3). Means followed by a different letter are significantly different according to Fisher's LSD test at α=0.05.

Since chlorophyll content of parsley, lettuce and broccoli decreased without obvious visual color shift, no relation between chlorophyll content and visually perceived color is evident. The effect of heat treatment on the vegetable homogenate samples on carotenoid content is shown in Figure B1 (Appendix B). Initial carotenoid content (NHT) was similar in all vegetable liquid samples (approximately 15 µg mL^{-1}), with the exception of lettuce samples (approximately 9 µg mL^{-1}). In contrast with the effect of heat treatment on chlorophylls, no changes were detected between samples before and after heat treatment, suggesting a minimal impact in carotenoid content as a result of thermal processing. Although this could suggest higher carotenoid stability in heated conditions when compared to chlorophylls, this

could also be a result of the inaccuracy of the method used for carotenoid determination, as previously mentioned.

4.3. Effect of pH and food additives on the stability of color and chlorophyll after pasteurization

The effect of various treatments on the heat-stability of 10% vegetable homogenates was studied to determine the influence of sodium bicarbonate ($NaHCO_3$) and zinc chloride ($ZnCl_2$) and that of pH (4.5, 6.0 and 8.5) on the quality of these samples. To facilitate discussion, sample identification is shown in Table 5. Control samples (CT) are the vegetable homogenates at native pH, without food additive or pH adjustment.

Table 5 - Design matrix and sample ID for pH and food additive experiments.

Sample ID	Treatment
SB_4.5	5 mg L^{-1} Na_2CO_3, pH 4.5
SB_6.0	5 mg L^{-1} Na_2CO_3, pH 6.0
SB_8.5	5 mg L^{-1} Na_2CO_3, pH 8.5
ZC_4.5	100 mg $L^{-1}ZnCl_2$, pH 4.5
ZC_6.0	100 mg L^{-1} $ZnCl_2$, pH 6.0
ZC_8.5	100 mg L^{-1} $ZnCl_2$, pH 8.5

Soluble solid content

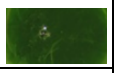
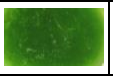
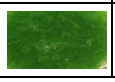
Soluble solid content of all samples before and after the heat treatment is summarized in Table A1 (Appendix A). In non-pasteurized samples, the addition of sodium bicarbonate increased the SSC from 0.7 to 1.2. The concentration of zinc chloride was low and did not change SSC value, maintaining the SSC of control samples (0.5, 0.2 and 0.7% for spinach, parsley and broccoli samples, respectively).

The effect of heat treatment on the SSC of homogenates with food additives was small and inconsistent: while in some samples heat treatment led to an increase in SSC (e.g. in ZC_ 6 for spinach samples, from 0.4 to 0.6%) in other were registered decreases (e.g. SB_8.5 in parsley samples, from 1.2 to 0.7%) or maintenance (e.g. ZC_4.5 spinach samples). These differences are small and tentatively attributed to experimental error. Nonetheless, further research into the ion exchange capacity of the different matrices, due to differences in the density of negative charges in cell wall materials (Shomer et al., 2003) may turn out to explain the differential effects of salts in the refractometric index of the different homogenates.

Color

Table 6 shows the effect of heat treatment on homogenate samples with sodium bicarbonate addition. Control (CT) samples were included for comparison with homogenates with salts and adjusted pH.

Table 6 – Effect of sodium bicarbonate on hue and visual color of samples without (NHT) and with (HT) heat treatment. Values are means ± s.d. (*n*=3). Means followed by a different letter are significantly different according to Fisher's LSD test at α=0.05 (within columns).

Salt and pH treatment	Heat treatment	Spinach		Parsley		Broccoli	
		hue angle	Photo record	hue angle	Photo record	hue angle	Photo record
CT	NHT	129.3^{d}±1.0		132.7^{e}±0.8		132.8^{e}±0.1	
	HT	125.3^{c}±0.5		125.7^{d}±0.5		126.4^{e}±0.2	
SB_4.5	NHT	129.1^{d}±1.0		131.2^{c}±0.5		132.0^{d}±0.1	
	HT	104.3^{b}±0.2		102.6^{b}±0.8		107.4^{c}±0.7	
SB_6.0	NHT	130.3^{d}±0.7		133.7^{e}±0.8		131.7^{d}±1.5	
	HT	129.9^{d}±1.7		131.2^{c}±0.5		133.0^{b}±0.2	
SB_8.5	NHT	129.0^{d}±0.3		133.5^{e}±0.8		131.7^{d}±0.5	
	HT	137.9^{a}±1.2		136.5^{a}±0.5		137.2^{a}±0.2	

The addition of sodium bicarbonate had a marginal effect on hue and visually perceived color of samples without heat treatment (Table 6). However, after heat treatment, the presence of sodium bicarbonate maintained or increased the hue angle in relation to CT samples. This maintenance or increase of the green hue is clearly observed in the photographic records (Table 6).

Sodium bicarbonate effect on pasteurized vegetable juices was dependent on the pH of the homogenate: hue decreased by 18% in heat-treated samples containing sodium bicarbonate at pH 4.5 in relation to pasteurized control samples. This decrease in hue angle had a significant impact on perceived color changing it from the originally perceived green to olive green/brown (Table 6).

At pH 8.5 and 6.0, sodium bicarbonate had an overall positive effect on visually perceived color and hue sample value, compared to control samples. This effect was more evident at pH 8.5, with hue angle increases of 8 and 4% in all SB_8.5 and SB_6 samples, respectively. Perceived color of SB_8.5 samples varied according to the raw material. Spinach and parsley

homogenates after pasteurization had more intense and brighter greens, while pasteurized broccoli samples were darker in comparison.

Many factors can explain hue and color stabilization in samples with added sodium bicarbonate in slightly acidic to alkaline conditions (pH 6.0 and 8.5). Although the presence sodium bicarbonate can lead to the formation of sodium chlorophyllin complexes (Srilakshmi, 2003), there can also be chlorophyll conversion into chlorophyllides (Zheng et al., 2014). Both molecules are greener and less heat labile than chlorophyll. High pH also stabilizes chlorophyll by itself, since chlorophyll stability decreases with higher H^+ concentration in the medium, reducing probability of pheophytinization. (Andrés-Bello et al., 2013; Zheng et al., 2014).

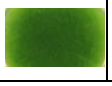
pH strongly affects color, as seen in SB_4.5 samples: despite the positive effect of sodium bicarbonate in the hue and visual color of samples at higher pH, this effect is overwritten by the low pH of the medium. As a consequence of this low pH, chlorophyll is converted to pheophytins, which is accompanied with a color shift from bright green to olive green (Steet & Tong, 1996), irrespective of the presence of sodium bicarbonate.

The initial intensity of color also had an effect on color perception after the process. Vegetable homogenates with a more intense initial color (such as in broccoli samples) had better color retention with heat treatment than the lighter and less intense greens (spinach and parsley samples), even at higher pH values. Although there was a consistent effect of sodium bicarbonate and pH on the hue angle, the relationship between sample hue and perceived color was not strong. For instance, pasteurized SB_8.5 samples of spinach and broccoli had similar hue angles (137°) but visually perceived color was significantly different with spinach samples brighter than those of broccoli. Differences in L* and C* color parameters for similar hues justify the different visual perception of colors. For spinach SB_8.5, L* and C* values were 22.4 and 5.2 while for broccoli SB_8.5 values of 28.0 and 10.5 were determined (Table A2, Appendix A). Although small, differences in these values for both samples can account to differences in visual color. Higher L* and C* values in broccoli SB_8.5 samples explain its higher visually perceived color saturation as well as lower luminosity, and darker green of these samples when compared to SB_8.5 of spinach samples. Other aspects of visual perception such as brightness, cannot be measured with the tristimulus colorimeter.

Table 7 shows the effect of heat treatment in homogenate samples with zinc chloride at different pH values.

Zinc chloride addition to samples before heat treatment had a similar effect to that of sodium bicarbonate, with no obvious differences observed in visual color or hue angle, compared to control samples, irrespective of plant material.

Table 7 - Effect of zinc chloride on hue and visual color of samples without (NHT) and with (HT) heat treatment. Values are means ± s.d. (*n*=3). Means followed by a different letter are significantly different according to Fisher's LSD test at α=0.05 (within columns).

Salt and pH treatment	Heat treatment	Spinach		Parsley		Broccoli	
		hue angle	Photo record	hue angle	Photo record	hue angle	Photo record
CT	NHT	129.3^{f}±1.0		132.7^{d}±0.8		132.8^{f}±0.1	
	HT	125.3^{e}±0.5		125.7^{c}±0.5		126.4^{f}±0.2	
ZC_4.5	NHT	128.2^{fd}±1.0		131.5^{d}±0.2		133.3^{b}±0.2	
	HT	106.6^{c}±0.4		108.2^{b}±0.1		114.6^{d}±0.6	
ZC_6.0	NHT	129.1^{f}±0.8		132.7^{d}±1.7		132.6^{e}±1.0	
	HT	123.6^{b}±0.9		123.7^{a}±0.8		128.1^{c}±0.2	
ZC_8.5	NHT	127.5^{d}±1.9		132.2^{d}±0.1		132.3^{e}±0.6	
	HT	137.3^{a}±0.7		132.3^{d}±0.6		134.5^{a}±0.3	

Like sodium bicarbonate-treated samples, zinc chloride added at pH 8.5 improved green color perception, increased color intensity of pasteurized samples and induced higher hue values regarding respective control sample. Hue increased 9% in spinach samples, 6% in broccoli samples and 5% in parsley samples with zinc chloride addition, while at pH 6.0 and 4.5 there was an overall decrease in hue (4% for spinach, 7% for parsley and 3% for broccoli, at pH 6, and 15% for spinach, 14% for parsley and 9 % for broccoli, at pH 4.5). The effect of zinc chloride at higher pH values are likely due to the formation of zinc-chlorophyll complexes, which improve green color and are also more stable to heat and to acidic conditions than chlorophyll (Zheng et al., 2014). However, as previously mentioned for sodium bicarbonate samples, medium pH plays an important part in the color of samples. The effect of zinc chloride at lower pH values (4.5) is negligible when compared to possible pheophytin production and consequential color shift. Once again, no clear relation between was observed between hue and visually perceived color of samples, since the same hue value often corresponds to different visually perceived colors. For example, in the case of non-pasteurized ZC_8.5 samples, although parsley and broccoli appear visually different, they have similar hue values (132°). An analysis of L* and C* values of these samples shows that, although similar in value (parsley L* value is 27.4 and C* value is 8.8, while broccoli L* value is 26.1 and C* value is

8.0), slight changes lead to observable differences in visually perceived color of homogenate samples. As it can be seen in Table 7, the visually perceived color of spinach, broccoli and parsley samples was similar, regarding the same pH condition.

When using zinc chloride as food additive, the influence of plant material on perceived color is diminished, especially after pasteurization. Zinc chloride addition to samples before heat treatment had a similar effect to that of sodium bicarbonate, with no obvious differences observed in visual color or hue value, compared to control samples, irrespective of plant material.

Overall, both sodium bicarbonate and zinc chloride are effective additives to stabilize the green color of the homogenates at pH 6 and above, although sodium bicarbonate was able to maintain chlorophyll stable at 6.0 better than zinc chloride, irrespective of raw material. Nonetheless, in acidic conditions both food additives were ineffective to prevent color change.

Chlorophyll a and chlorophyll b content

The effect of sodium bicarbonate and zinc chloride on chlorophyll a content of spinach, parsley and broccoli leaves homogenates at different pH values is shown in Figure 13. pH was the most important variable affecting not only the hue angle and color, but also chlorophyll content. Chlorophylls were more stable at higher pH values (Figure 13). The addition of sodium bicarbonate and zinc chloride had improved the retention of chlorophyll concentration, although their effect was pH dependent.

All sodium bicarbonate-treated samples at pH 8.5 had higher chlorophyll *a* content than control samples, irrespective of heat treatment and plant material. Unpasteurized broccoli samples at pH 8.5 and 6.0 had higher concentration of chlorophyll *a*: at pH 8.5 chlorophyll *a* content was 123.4 and 103.6 μg mL^{-1}, and at pH 6.0 chlorophyll *a* content was 119.5 and 108.8 μg mL^{-1}, for sodium bicarbonate and zinc chloride samples, respectively. Samples of parsley treated with sodium bicarbonate at pH 8.5 had the second highest concentration (109.2 μg mL^{-1}), followed by spinach samples (81.1 μg mL^{-1}).

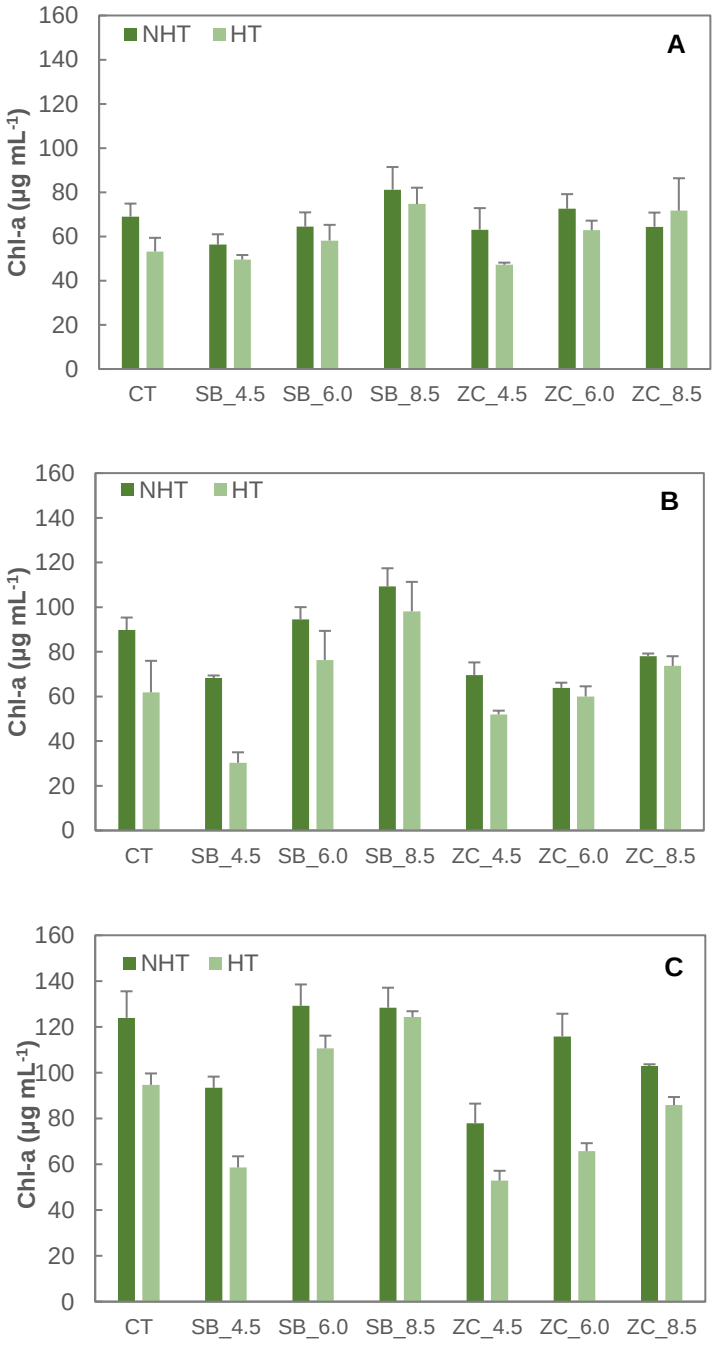


Figure 13 - Heat treatment, food additive and pH effects on chlorophyll *a* content of vegetable homogenates: spinach (A), parsley (B) and broccoli (C). Error bars are standard deviation (*n*=3). Means followed by a different letter are significantly different according to Fisher's LSD test at α=0.05.

The addition os sodium bicarbonate increased significantly (P<0.05) the chlorophyll content of samples at pH 8.5, in all raw material homogenates. At pH 8.5 and and 6.0, sodium bicarbonate also reduced the loss of chlorophyll *a* due to heat treatment in all samples: from 23%, 31% and 24% of chlorophyll *a* losses in control samples of spinach, parsley and broccoli, respectively, to 8 and 10%, 19 and 8% and 14 and 3% losses in SB_6.0 and SB_8.5, for

spinach, parsley and broccoli, respectively. Spinach samples with sodium bicarbonate at pH 4.5 had a similar retention of chlorophyll (88%) with pasteurization compared to control samples (77%), while in parsley and broccoli treated with sodium-bicarbonate at pH 4.5 chlorophyll a decreased 55 and 37%, respectively.

The effect of zinc chloride was pH dependent, but varied more with raw material, than that of sodium bicarbonate. In spinach samples, chlorophyll *a* content of unheated homogenates did not change with addition of zinc chloride, as it did with sodium bicarbonate, irrespective of pH. Spinach homogenates treated with zinc chloride had similar chlorophyll *a* content to control samples, while in parsley and broccoli the samples with added zinc chloride had lower chlorophyll *a* content than control samples.

Spinach and parsley homogenates treated with zinc chloride had similar retention of chlorophyll *a* with heat treatment for all pH values. Chlorophyll *a* retention in broccoli samples was lower than in spinach and parsley samples, although the effect of pH on chlorophyll *a* retention was not clear in this case: although higher chlorophyll retention is observed at pH 8.5 (83%), as would be expected, the second highest retention was observed in zinc chloride treated samples ta pH 4.5 (68%), followed by those at pH 6.0 (57%).

Overall, sodium bicarbonate was more effective than zinc chloride in maintaining higher levels of chlorophyll *a* before and after the heat treatment, and at lower pH (6.0). Sodium bicarbonate was also more effective than zinc chloride when compared with control samples, and led to higher chlorophyll content in most of samples in study. The pH value contributed more to differences in chlorophyll *b* content in samples ($P<0.05$) compared to food addives and heat treatment.

Figure 14 shows the effect of sodium bicarbonate and zinc chloride on chlorophyll *b* content of spinach, parsley and broccoli homogenates at different pH values.

Overall, spinach samples had lower initial chlorophyll *b* content, for all food additives and pH considered, followed by parsley and broccoli samples. Chlorophyll *b* in spinach samples did not change with the food additives and pH adjustment. In unpasteurized parsley samples at pH 6.0 and 8.5, the addition of sodium bicarbonate increased chlorophyll *b* content by 15 and 33%, respectively, compared to chlorophyll *b* content of samples without sodium bicarbonate addition (control samples). Chlorophyll *b* content of non-heat treated parsley samples with addition of sodium bicarbonate at pH 8.5 were significantly higher ($P<0.05$) than the rest of parsley homogenate samples.

Sodium bicarbonate addition in spinach samples did not affect significantly ($P<0.05$) the chlorophyll *b* content before or after pasteurization. Parsley samples with sodium bicarbonate at pH 4.5 had higher losses in chlorophyll *b* content than the control, although the effect of pH 6.0 and 8.5 was different than expected: chlorophyll *b* losses in control samples were of 38%, followed by samples with sodium bicarbonate at pH 4.5 (64%), samples with sodium

bicarbonate at pH 6.0 (31%), and the largest chlorophyll *b* losses in samples with sodium bicarbonate at pH 8.5 (43%).

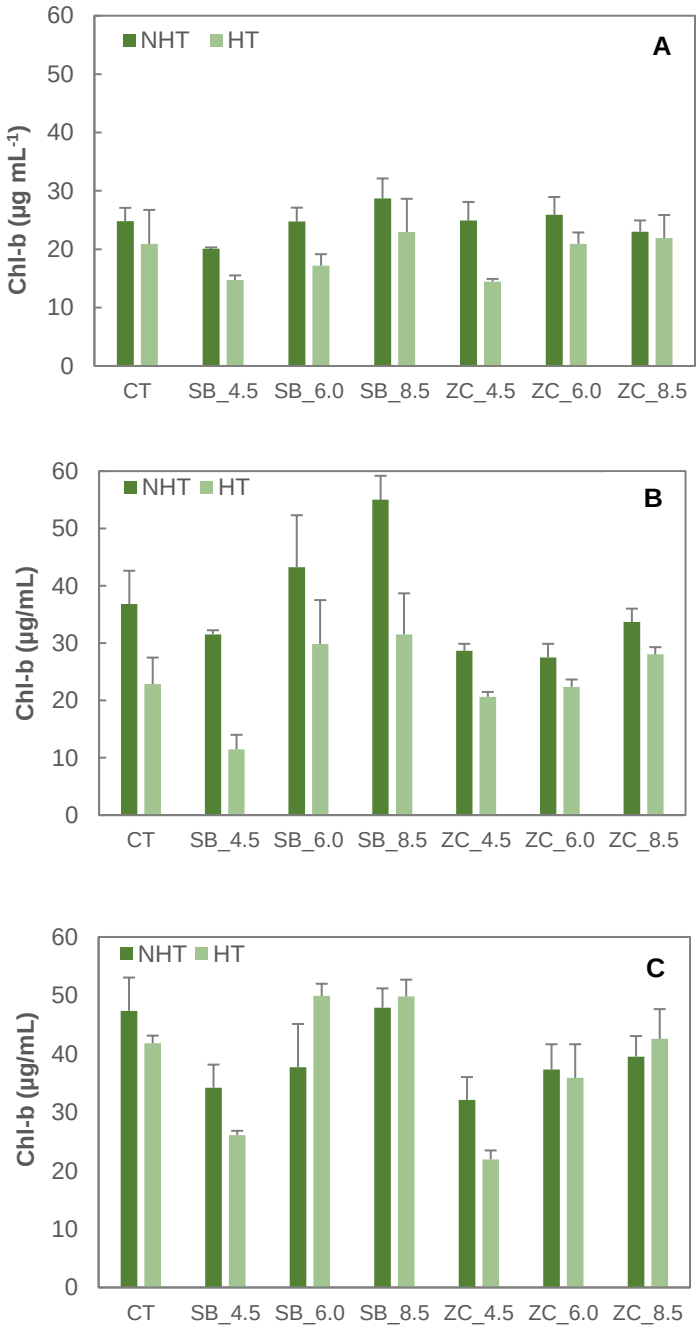


Figure 14 - Heat treatment, food additive and pH effects on chlorophyll b content of vegetable homogenates: spinach (A), parsley (B) and broccoli (C). Error bars are standard deviation (*n*=3). Means followed by a different letter are significantly different according to Fisher's LSD test at α=0.05.

In broccoli homogenate samples, sodium bicarbonate induced an significant (P<0.05) increase of chlorophyll *b* content in pasteurized sodium bicarbonate samples at pH 8.5 of 24%, compared to sample without heat treatment. Since there is no chlorophyll metabolism in the system, the higher absorbance readings may result from interference with other compounds

such as chlorophyllides and sodium chlorophyllin complexes formed due to the action of pH and sodium bicarbonate presence. Broccoli samples no great difference was registered for the effect of zinc chloride and sodium bicarbonate on chlorophyll *b*, for all samples, irrespective of pH.

The addition of zinc chloride to parsley and broccoli samples did not affect chlorophyll *b* content in unheated samples. The addition of this additive did not make a great difference in chlorophyll *b* retention on all vegetable homogenate samples, for pH 8.5 and 6.0. Chlorophyll *b* losses in zinc-treated samples were greater at pH 4.5, compared to those registered at pH 6.0 and 8.5. At pH 4.5 chlorophyll *b* losses were of 28% in spinach, 32% in broccoli and 42% in parsley.

Total chlorophyll content

Figure 15 summarizes the effect of sodium bicarbonate and zinc chloride on total chlorophylls content of spinach, parsley and broccoli homogenates at different pH values.

The effect of sodium bicarbonate and zinc chloride on total chlorophyll concentration was similar to their effect on chlorophyll *a* and chlorophyll *b*, previously described. Total chlorophylls of spinach samples changed only slightly with added zinc chloride and sodium bicarbonate irrespective of pH value. Sodium bicarbonate addition had a positive effect on chlorophylls content of parsley and broccoli samples at 8.5 and 6.0, compared to controls. In broccoli homogenates sodium bicarbonate preserved the initial total chlorophyll content, while at pH 4.5 both samples had lower chlorophyll concentration than the control, before and after heat treatment.

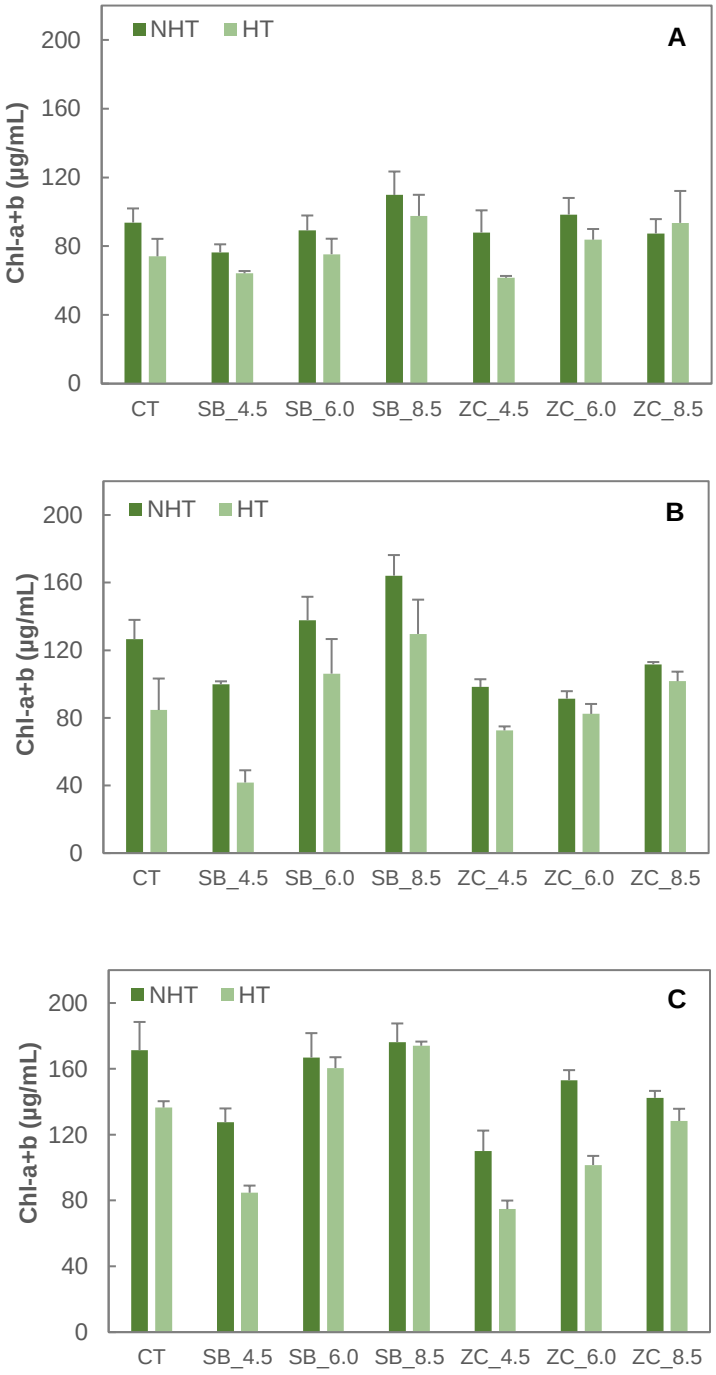


Figure 15 - Heat treatment, food additive and pH effects on total chlorophylls content of vegetable homogenates: spinach (A), parsley (B) and broccoli (C). Error bars are standard deviation (*n*=3). Means followed by a different letter are significantly different according to Fisher's LSD test at α=0.05.

In parsley, zinc chloride at pH 8.5 and 6.0 helped to maintain chlorophylls content of unpasteurized samples. Broccoli had an overall higher initial chlorophylls content followed by spinach and parsley. Sodium bicarbonate and zinc chloride had a greater effect on chlorophyll retention at higher pH values (8.5 and 6.0) than at lower values (4.5), with sodium bicarbonate

registering greater effects in chlorophyll maintenance than zinc chloride, especially at pH 8.5, where sodium bicarbonate allowed a decrease in chlorophylls loss compared to controls.

Carotenoid content

The effect sodium bicarbonate and zinc chloride on carotenoid content of spinach, parsley and broccoli homogenates at different pH is summarized in Figure B2 (Appendix B).

The addition of sodium bicarbonate increased initial (NHT) carotenoid content of homogenates at pH 8.5, in all four raw materials, while at pH 6.0 increased initial carotenoid content only in parsley homogenate samples. Heat treatment did not affect overall carotenoid content of samples, irrespective of plant material. Zinc chloride did not have an effect on initial carotenoid content, nor the maintenance of carotenoid content with heat treatment.

pH had a similar effect on carotenoid content as was observed with chlorophyll, with decreasing concentration with decreasing pH.

4.4. Effect of pH and food additives on quality of heat treated homogenates during shelf-life

After initial characterization and pasteurization, the vegetable homogenates were stored at 20 °C and analysed for pH, SSC, color, and chlorophyll content during a 16-day shelf-life period for spinach and parsley samples and 11-day period for broccoli.

pH and soluble solid content

Figure 16 shows the changes pH during shelf-life of spinach, parsley and broccoli homogenates, as affected by used food additive (sodium bicarbonate vs zinc chloride) at pH 4.5, 6.0 and 8.5.

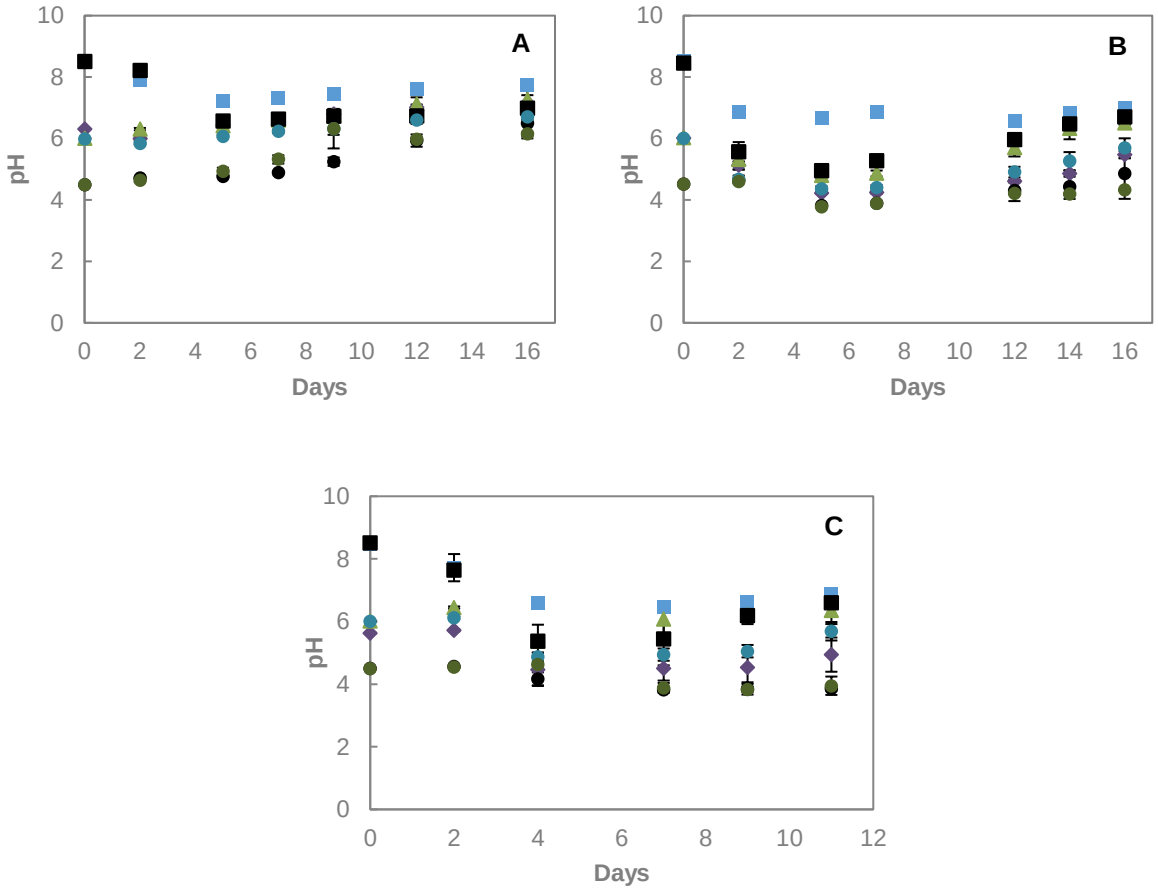


Figure 16 - pH evolution during shelf-life for spinach (A), parsley (B) and broccoli (C) homogenates; ◆- CT; ■- SB_8.5; ▲- SB_6; ● - SB_4.5; ■ - ZC_8.5; ● - ZC_6; ● - ZC_4.5. Error bars are standard deviation (*n*=3).

When treated with the same additive and at the same initial pH, the changes in pH followed a similar trend in all vegetable liquid matrices. During shelf-life, sample pH from spinach, parsley and broccoli samples decreased at pH 8.5, irrespective of food additive. In

samples containing sodium bicarbonate with an initial pH of 8.5, pH values decreased to 7.8 in spinach samples, to 7.0 in parsley, and to 6.9 in broccoli homogenates. In homogenates treated with zinc chloride, pH decreased from 8.5 to 7.0 in spinach, 8.4 to 6.7 in parsley and 8.5 to 6.6 in broccoli. In homogenates with initial pH adjusted to 4.5 and 6.0, a general trend for pH maintenance and slight increase was observed, respectively. It is noteworthy that after 4-5 days of shelf-life the sample pH tended to the initial raw material pH (6.3 for spinach, 6.0 for parsley, 5.6 for broccoli), irrespective of initial homogenate sample pH.

Soluble solid content remained constant (~1.0% for samples with added $NaCO_3$ and ~ 0.5% for remaining samples) during shelf-life (Figure A1, Appendix A). Higher soluble solids content were registered in homogenates containing sodium bicarbonate in relation to controls. The addition of zinc chloride to the liquid samples had no influence on soluble solids content which was similar to that of control samples (~0.5%) (Figure A1, Appendix A).

Color

The changes in hue angle during shelf-life of spinach, parsley and broccoli homogenate under the influence of food additive and pH are shown in Figure 17.

In general, samples with an initial pH of 8.5 samples (SB_8.5 and ZC_8.5) had higher hue angle than control samples, in all raw materials (approximately 124° for spinach, 108° for parsley and 132.6° for broccoli) followed by samples at pH 6 (SB_6 and ZC_6). Samples at pH 4.5 (SB_4.5 and ZC_4.5), irrespective of food additive and plant material, had the lowest hues (99.0°, 98.5° and 106.3°, for spinach, parsley and broccoli respectively). At the same initial pH value of 4.5, homogenates containing zinc chloride had higher hue angles than those with sodium bicarbonate. Moreover, pH 4.5 homogenates had an initial brownish color rather than the target green. This observation points out to a negative color deviation from the target green when the pH is adjusted to 4.5 (Table C1-C3, Appendix C).

Between the 2nd and 5th day, parsley samples had a different behaviour that spinach samples for pH 8.5: while spinach samples with sodium bicarbonate and zinc chloride had similar hue decrease patterns, in parsley samples there was a sudden decrease of hue in SB_8.5, when compared to ZC_8.5. Broccoli samples had a slower decrease in hue (as seen in Figure 17C) which could also be observed visually as a slower decrease in green color intensity was registered (Table C3, Appendix C).

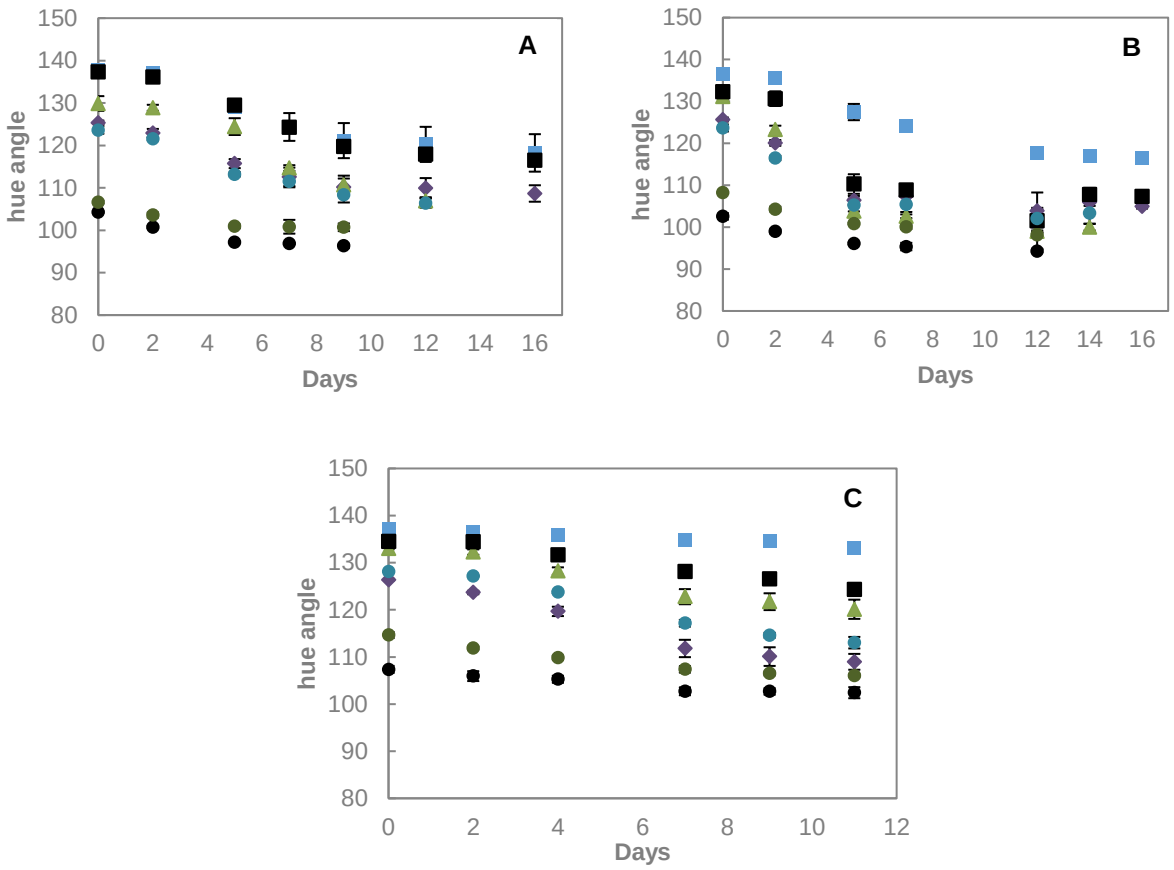


Figure 17- Hue angle evolution during shelf-life for spinach (A), parsley (B) and broccoli (C) homogenates; ◆- CT; ■- SB_8.5; ▲- SB_6; ● - SB_4.5; ■ - ZC_8.5; ● - ZC_6; ● - ZC_4.5. Error bars are standard deviation (*n*=3).

In broccoli distinct patterns are observed (Figure 17C): at pH 8.5, hue angle changed at a lower rate than at pH of 6.0 and 4.5. However, and as previously mentioned, broccoli samples at pH 4.5 had a significant deviation from the target green color. The hue of the homogenates was better retained in the presence of sodium bicarbonate than zinc chloride.

Independently of the initial pH and for all raw materials, green hues were better retained with sodium bicarbonate. The color of sodium bicarbonate-treated homogenates at pH 8.5 were perceived as brighter green throughout shelf-life (Table C1-C3, Appendix C). Although the addition of sodium bicarbonate and zinc chloride had different impacts on vegetable sample color, pH adjustment seems to be the more influent factor on samples overall color, irrespective of the vegetable used for homogenate production.

Chlorophyll a and chlorophyll b content

Figure 18 shows the changes in chlorophyll *a* and chlorophyll *b* of spinach, parsley and broccoli samples under the influence of food additive and adjusted pH during shelf-life.

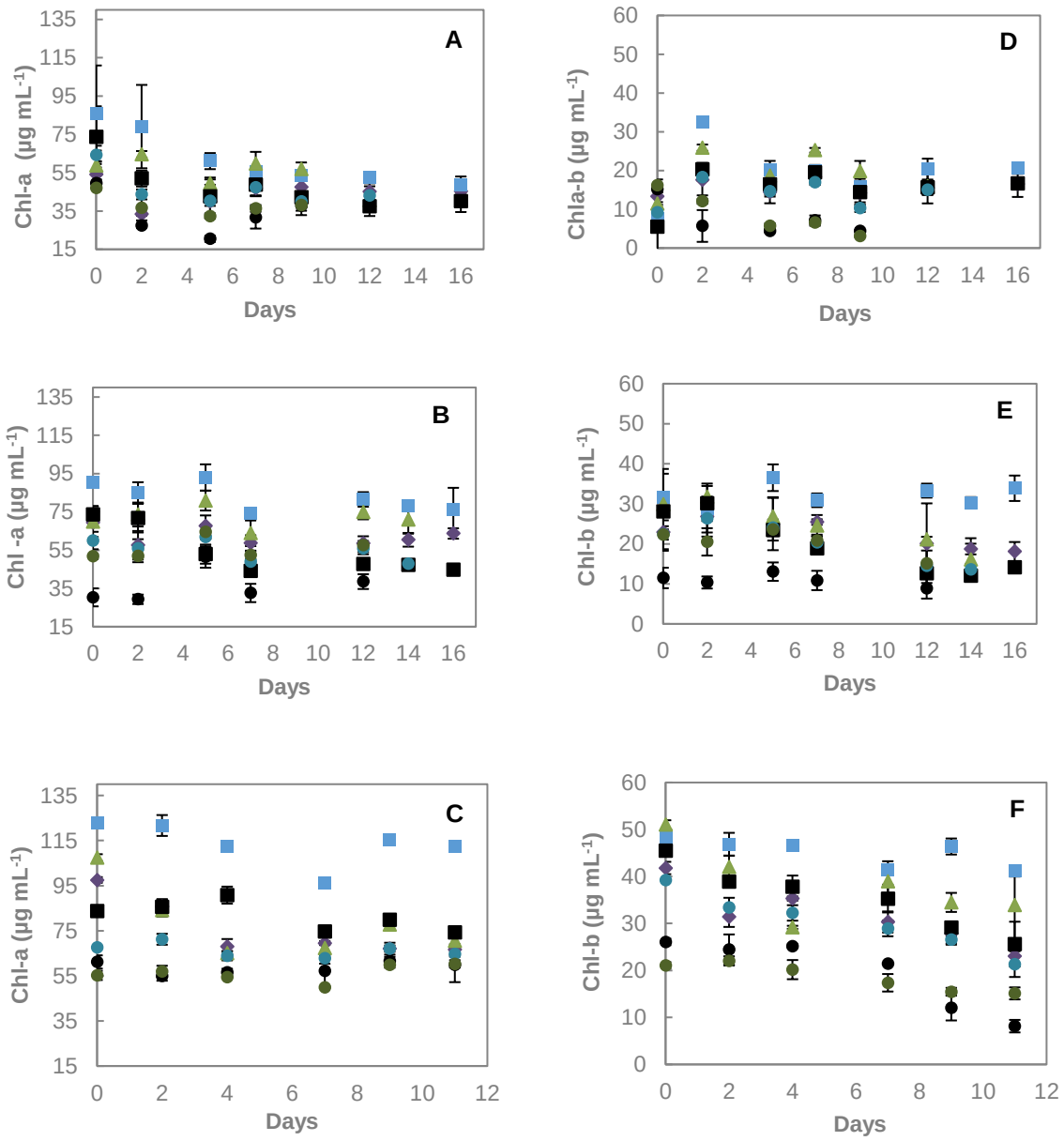


Figure 18 – Chlorophyll *a* and chlorophyll *b* evolution during shelf-life for spinach (A,D), parsley (B,E) and broccoli (C,F); ◆- CT; ■- SB_8.5; ▲- SB_6; ● - SB_4.5; ■ - ZC_8.5; ● - ZC_6; ● - ZC_4.5. Error bars are standard deviation (*n*=3).

Changes in chlorophyll *b* concentration during shelf-life showed a similar trend in spinach and parsley homogenates: in both samples, chlorophyll *b* content was maintained during shelf-life. In broccoli samples, chlorophyll *b* remained constant during the first 4 days, after which

time there was a decrease in concentration, except in homogenates containing sodium bicarbonate at pH 8.5, where chlorophyll *b* content remained constant.

In samples treated with sodium bicarbonate a clear relationship between pH and chlorophyll *a* and chlorophyll *b* content was found: the higher the sample pH, the higher the content of chlorophyll *a* and chlorophyll *b*. Despite their lower chlorophyll contents, homogenates with zinc chloride exhibited a similar behaviour to sodium bicarbonate samples regarding the influence of pH on chlorophyll content.

Differences between treatments were harder to discern in spinach samples, since after the 12th day all chlorophyll *a* and chlorophyll *b* values tended to the a value of approximately 43 µg mL^{-1} homogenate for chlorophyll *a* and 16 µg/ml chlorophyll *b*. This response was only observed in spinach samples.

Total chlorophylls content

Figure 19 presents the evolution of total chlorophylls content in spinach, parsley and broccoli homogenates with added sodium bicarbonate and zinc chloride at different pH values.

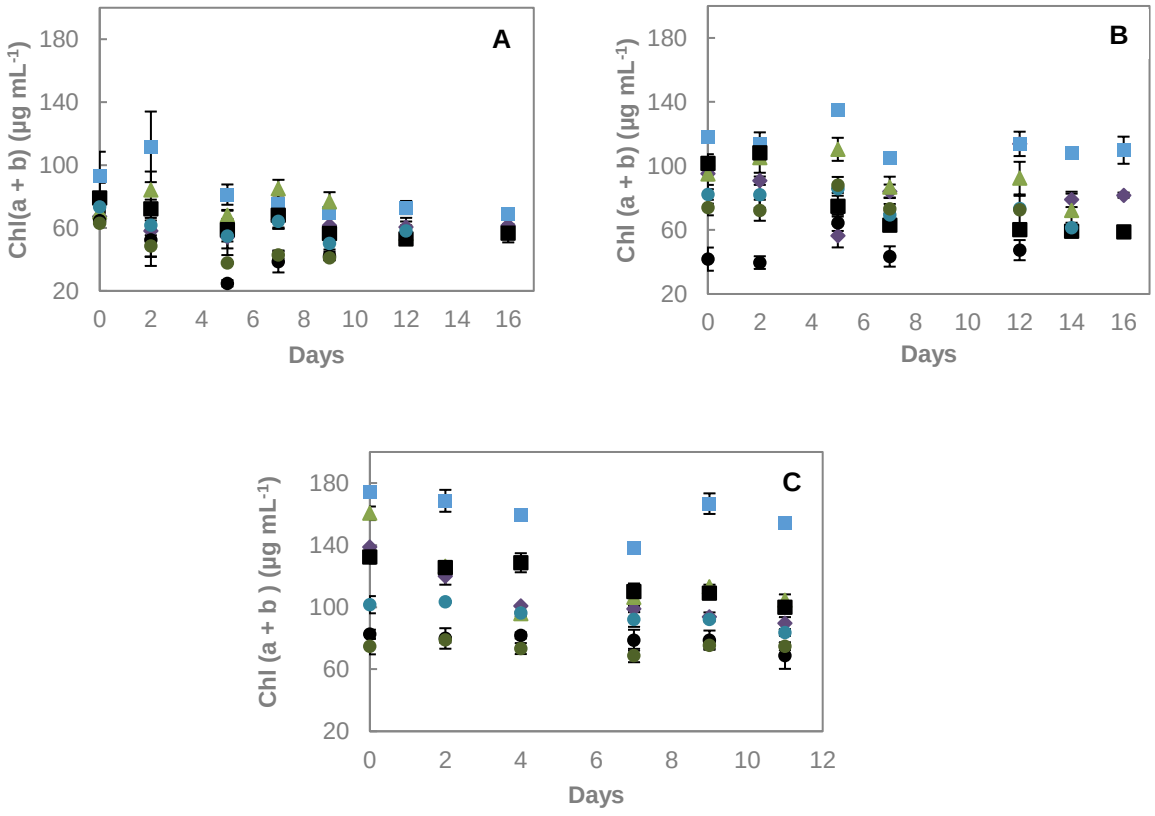


Figure 19 – Total chlorophylls evolution during shelf-life for spinach (A), parsley (B) and broccoli (C); ◆- CT; ■- SB_8.5; ▲- SB_6; ● - SB_4.5; ■ - ZC_8.5; ● - ZC_6; ● - ZC_4.5. Error bars are standard deviation (*n*=3).

The changes in total chlorophylls content were similar to those described for hue angle: higher pH (8.5) resulted in higher chlorophylls content in samples from all vegetable homogenates, while lower pH resulted in lower total chlorophyll content.

Nevertheless, plant material greatly influenced the total chlorophylls content during shelf-life: while spinach samples (all treatments) showed a more abrupt degradation with time, with a non-linear total chlorophylls evolution, parsley and broccoli had a more linear tendency indicating a slow degradation of total chlorophyll with time. Generally, broccoli samples had higher total chlorophyll content after pasteurization, maintaining higher values during shelf-life.

Carotenoid content

Carotenoid content did not change considerably during shelf-life (Figure B2, Appendix B). The addition of sodium bicarbonate had different effects depending of raw material. In spinach, samples SB_8.5 had the highest initial carotenoid content of all homogenate samples and treatments (33.7 µg mL^{-1}), with higher overall carotenoid content, compared to parsley and broccoli homogenate samples (all treatments). Carotenoid levels remained unaltered during shelf-life of parsley and broccoli samples, for all additives and pH values.

4.5. Influence of foliar application treatments on quality of heat treated homogenates

The lettuce homogenates produced as described in section 3.2 were used to evaluate the influence of pre-harvest zinc application and zinc chloride addition on various parameters. To facilitate discussion, the sample identification was used according to Table 8. Control samples (CT_CT) were set as fresh vegetable solutions at native pH, without pre-harvest zinc treatments or food additives.

Table 8 - Sample ID for foliar application treatments.

Sample ID	Treatment
CT_CT	No pre-harvest $ZnCl_2$/ No $ZnCl_2$ addition
CT_ZC	No pre-harvest $ZnCl_2$/ $ZnCl_2$ in addition
ZC_CT	Pre-harvest $ZnCl_2$/ No $ZnCl_2$ in addition
ZC_ZC	Pre-harvest $ZnCl_2$/ $ZnCl_2$ in addition

pH and soluble solid content

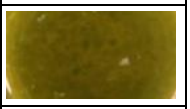
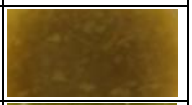
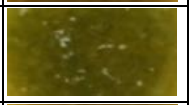
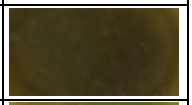
Results for pH, soluble solid content, color and photographic records of lettuce homogenate samples without (NHT) and with (HT) heat treatment are summarized in Table 9, for all performed treatments.

Initial pH and soluble solid content values for all samples were approximately 6.0 and 0.5%, respectively, and no change in these parameters was observed as a result of heat treatment (Table 9).

Color

Although no noteworthy difference was registered in sample hue, irrespective of pre-harvest treatments or additives ($P<0.05$), differences in color could be visually perceived as shown in Table 9. Less obvious was a slight visually perceived brightening in samples ZC_CT and ZC_ZC, which could be a result of zinc-chlorophyll complexes that could affect the homogenates green color. Overall C* value increased with heat treatment, pointing to a more saturated visual color, except in ZC_CT samples, where no significant difference ($P<0.05$) was registered before and after heat treatment.

Table 9 - pH, soluble solid content, color and photographic records of lettuce homogenate samples without (NHT) and with (HT). Values are means ± s.d. (*n*=3). Means followed by a different letter are significantly different according to Fisher's LSD test at α=0.05 (within columns).

Sample	Heat treatment	pH	SSC	Color			
				L*	C*	°hue	Photo Record
CT_CT	NHT	6.0^{f}±0.1	0.5^{ab}±0.1	25.3^{d}±0.1	4.5^{fe}±0.1	109.8^{c}±3.2	
	HT	5.8^{c}±0.1	0.5^{a}±0.1	27.3^{b}±0.1	6.1^{a}±0.4	109.8^{c}±3.2	
CT_ZC	NHT	5.9^{b}±0.1	0.4^{b}±0.1	25.8^{c}±0.1	5.0^{d}±0.1	112.2^{cb}±0.2	
	HT	5.7^{e}±0.1	0.5^{ab}±0.1	27.9^{a}±0.3	7.1^{a}±0.4	112.2^{cb}±0.2	
ZC_CT	NHT	6.0^{a}±0.1	0.5^{ab}±0.1	25.4^{d}±0.1	4.6^{f}±0.2	115.5^{a}±0.7	
	HT	6.0^{a}±0.1	0.5^{ab}±0.1	26.0^{c}±0.1	4.4^{fe}±0.1	115.5^{a}±0.7	
ZC_ZC	NHT	6.0^{a}±0.1	0.5^{ab}±0.1	25.5^{d}±0.2	4.8^{ef}±0.1	114.5^{ba}±0.7	
	HT	5.8^{d}±0.1	0.5^{ab}±0.1	27.0^{b}±0.1	5.4^{c}±0.2	114.5^{ba}±0.7	

Chlorophyll a and chlorophyll b content

In Figure 20 is summarized the effect of heat treatment on chlorophyll *a*, chlorophyll *b* and total chlorophyll content on lettuce homogenate samples under various treatments.

Contrary to what was reported by other authors (Fahad et al., 2014; Min et al., 2004), fertilization with zinc chloride did not increase chlorophyll content in the lettuce homogenates, although it did increase slightly the hue angle, in all samples However this lack of effect on chlorophyll on the lettuce homogenates could be due to different concentrations and different salts used by these authors (20 mg L^{-1} of $ZnSO_4$ for Fahad et al., and 1.2-1.4 mg L^{-1} of $ZnCl_2$ for Min et al.).

Chlorophyll content varied the most as a result of pasteurization due to chlorophylls sensitivity to heated conditions. Chlorophyll *a* content decreased approximately 27% in all samples. Chlorophyll b decreased the most in CT_ZC samples (13%), followed by CT_CT (8%) and ZC_CT (9%) samples. Following heat treatment, the chlorophyll *b* content in sample ZC_ZC was increased 20% regarding non-heat treated samples. However, this could be due

to various factors since no chlorophyll is being metabolized, including quantification and detection errors, heat degradation of other compounds present in the homogenates that could increase chlorophyll detection, as well as increases in chlorophyll readings due to the formation of zinc-chlorophyll complexes.

Total chlorophyll content decreased about 22% for all samples, except for sample ZC_ZC (16 %).

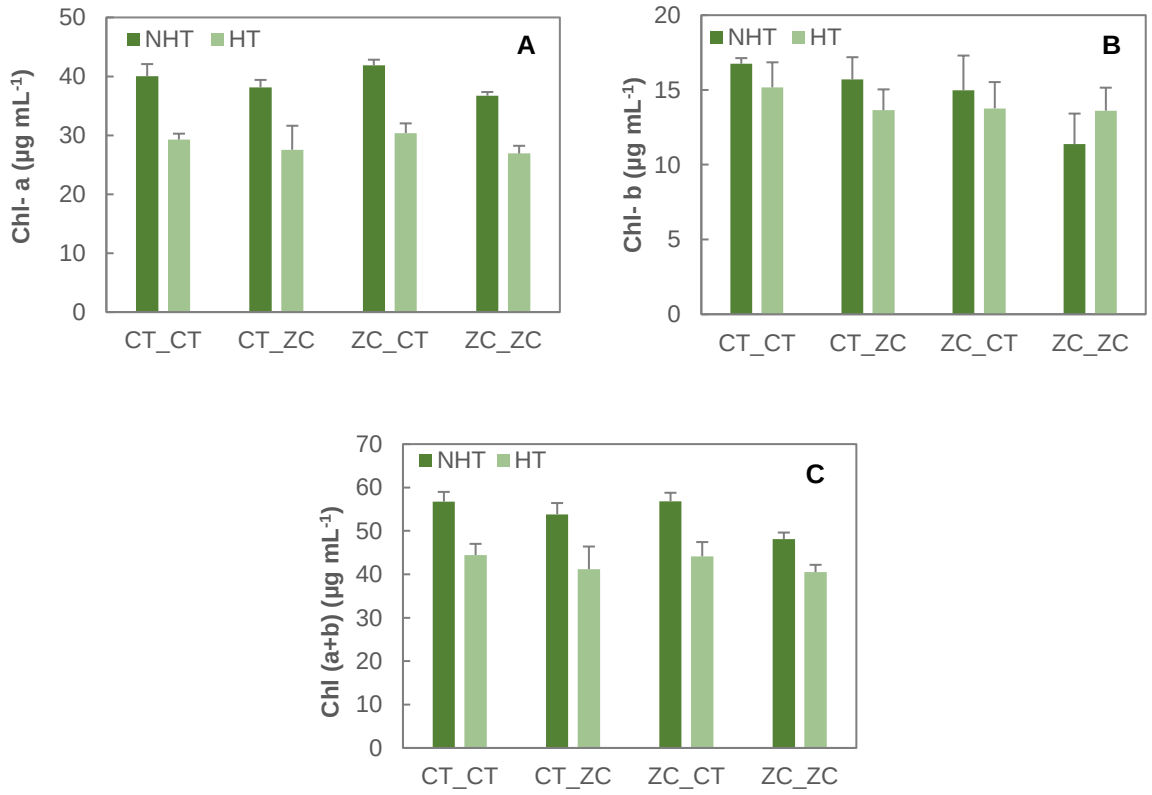


Figure 20 - Heat treatment effect on chlorophyll *a* (A), chlorophyll *b* (B) and total chlorophyll (C) content of lettuce homogenates. NHT – no heat treatment; HT – heat treatment. Error bars are standard deviation (*n*=3).

Overall, pre-harvest zinc application and use of zinc as an additive, combined or in separate, do not appear to have a significant ($P<0.05$) influence in chlorophyll (*a*, *b* and total) protection with heat treatment, as seen in Figure 20.

The effect of heat treatment in the carotenoid content of lettuce homogenate samples is shown in Figure 21.

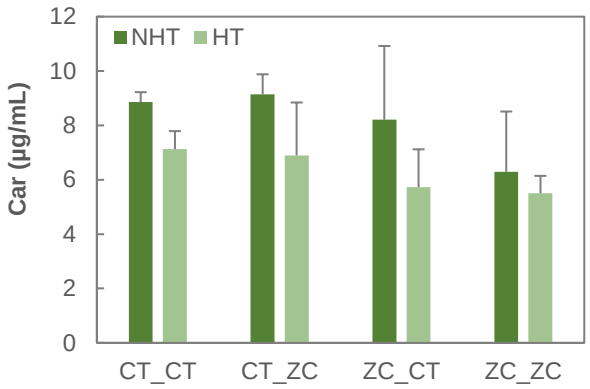


Figure 21 – Effect of heat treatment in carotenoid content of lettuce homogenates. NHT – no heat treatment; HT – heat treatment. Error bars are standard deviation (*n*=3).

Carotenoid losses resulting from thermal treatment were of 20, 25, 30 and 13 % for samples CT_CT, CT_ZC, ZC_CT and ZC_ZC, respectively. It is interesting to notice that after pasteurization and under the influence of zinc foliar application (ZC_ samples) carotenoid losses were minimized when zinc chloride was added to the vegetable homogenates (13% vs. 30%, ZC_CT vs. ZC_ZC samples, respectively). Therefore, although the effect on chlorophylls stability not noteworthy, pre-harvest zinc application has a positive effect on carotenoids, which could be due to zinc ion influences in the main structure of carotenoids, leading to the formation of carotenoid zinc complexes with higher heat resistance.

4.6. Influence of foliar application treatments on quality of heat treated homogenates during shelf-life

Figure 22 shows the effect of pre-harvest zinc chloride applications changes in pH and SSC of lettuce homogenates during shelf-life.

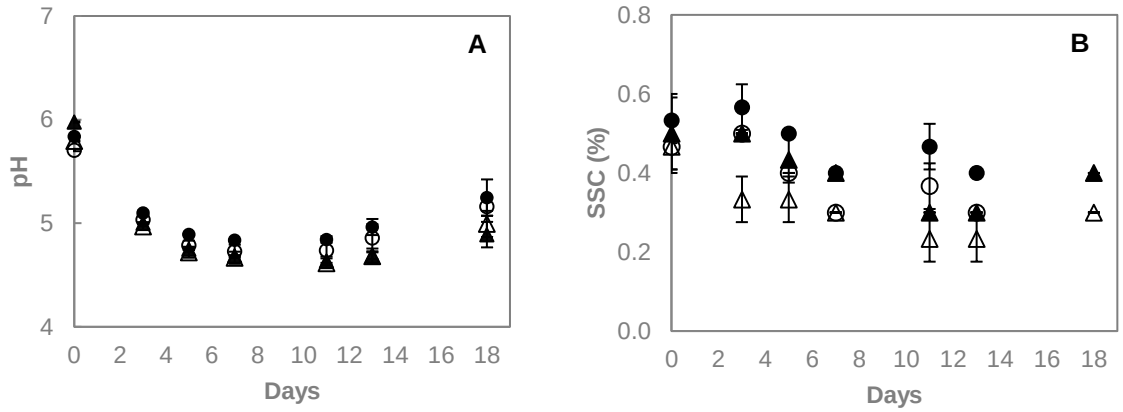


Figure 22 –Evolution of pH (A) and soluble solid content (B) during shelf-life of lettuce homogenates. ● - CT_CT; ○- CT_ZC; ▲ - ZC_CT; △ - ZC_ZC. Error bars are standard deviation (*n*=3).

pH decreased during the first 6 d of shelf-life (from ~6.0 to ~4.5), followed by an increase towards the last days of shelf-life reaching final values ~5.0 pH units (Figure 22A). This pattern was observed in all treatments. Soluble solid content (Figure 22B) ranged between 0.2% and 0.6% in all samples, and were relatively constant during the 18-d shelf life period.

The evolution of chlorophyll *a* and chlorophyll *b* content of lettuce homogenates during shelf-life is summarized in Figure 23. Chlorophyll *a* content of pasteurized lettuce homogenates ranged from 27.5 μg mL^{-1} (in CT_ZC samples) to 30.4 μg mL^{-1} (in ZC_CT samples). During shelf-life, similar degradation patterns were found among the treatments, with an initial decrease in chlorophyll *a* content until day 5 (from ~28.5 μg mL^{-1} to ~18 μg mL^{-1}) after which time the values remained constant until day 18. Chlorophyll *b* decreased in the first 3 days to about half the initial values (Figure 23B).

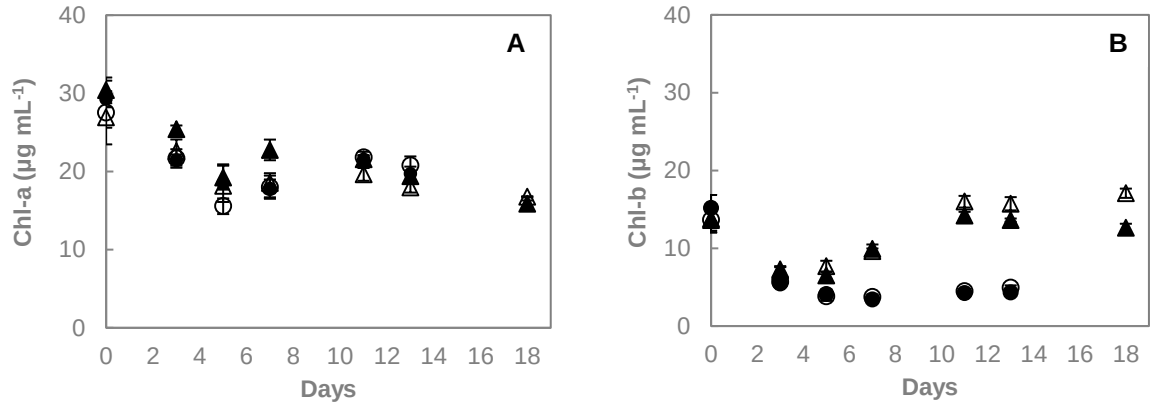


Figure 23 – Evolution of chlorophyll *a* (A) and chlorophyll *b* (B) content during shelf-life of lettuce homogenates. ● - CT_CT; ○- CT_ZC; ▲ - ZC_CT; △ - ZC_ZC. Error bars are standard deviation (*n*=3).

From that point on, distinct behaviours for samples with (ZC_CT and ZC_ZC) and without (CT_CT and CT_ZC) pre-harvest treatment were observed: chlorophyll *b* concentration in samples with pre-harvest foliar zinc application showed an increasing tendency, while those without pre-harvest foliar treatments experienced a decrease in chlorophyll b content, following what appears to be a first order degradation rate for chlorophyll degradation. This agrees with the conclusions of Ahmed et al. (2013), Canjura et al. (1991) and Gaur et al. (2006). It is apparent that foliar treatments had a greater effect on chlorophyll *b* concentration than chlorophyll *a*. The determined low chlorophyll *a* content of lettuce liquid samples may be due to naturally occurring low concentration of chlorophyll in the raw material, which may cause observed maintenance of chlorophyll value.

Figure 24 summarizes the variation of hue and total chlorophyll of lettuce homogenate samples during shelf-life.

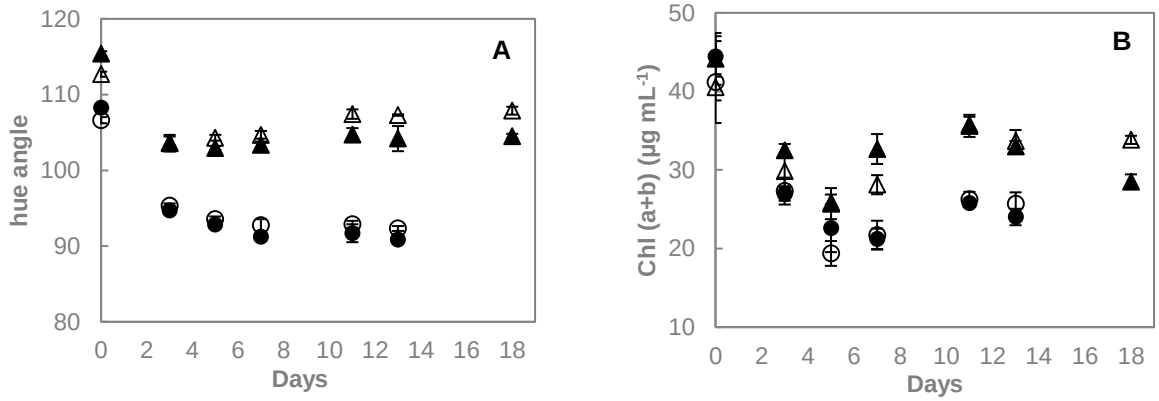


Figure 24 – Evolution of the hue angle (A) and total chlorophyll content (B) during shelf-life of lettuce homogenates. ● - CT_CT; ○- CT_ZC; ▲ - ZC_CT; △ - ZC_ZC. Error bars are standard deviation (*n*=3).

Hue and total chlorophylls changed during shelf-life according to similar patterns, which suggests a correlation between chlorophyll degradation and hue of lettuce vegetable samples, irrespective of treatment applied. Both samples with pre-harvest application of zinc show higher hue values, as well as higher total chlorophyll content, when compared to samples without pre-harvest zinc application. In both cases, a rapid initial decrease was observed, followed by a maintenance of these parameters until day 18. However, samples without foliar treatments had a faster decrease after the 3rd day of shelf-life in both chlorophyll (losses of 40% and 34% on CT_CT, CT_ZC samples compared to 26% losses in both ZC_CT and ZC_ZC samples) and hue (from 109.3 to 94.7 and 112.2 to 95.3 in CT_CT and CT_ZC samples, respectively, compared to 115.5 to 103.6 and 114.5 to 103.6 in ZC_CT and ZC_ZC samples). This observation suggests a positive effect of pre-harvest zinc application on chlorophyll content and hue maintenance of pasteurized lettuce homogenates during shelf-life, a fact that is also apparent in the visual color of these samples. Visually perceived color of lettuce with pre-harvest application of zinc chloride (ZC_CT and ZC_ZC) was darker than samples without treatment, and was better maintained during shelf-life (Table D1, Appendix D).

The variation of the ratio between chlorophyll *a* and chlorophyll *b* during shelf-life in lettuce homogenate samples is shown in Figure 25.

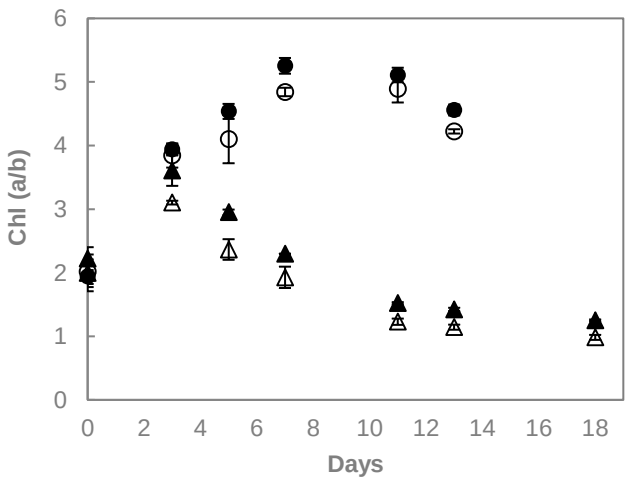


Figure 25 – Evolution of the ratio between chlorophyll a and chlorophyll b during shelf-life of lettuce homogenates. ● - CT_CT; ○- CT_ZC; ▲ - ZC_CT; △ - ZC_ZC. Error bars are standard deviation (*n*=3).

Theoretical ratio between chlorophyll *a* and chlorophyll *b in planta* is approximately 3 (Gaur et al., 2006) and since it is considered that chlorophyll *a* is the least stable of the two main vegetable chlorophylls, this ratio is expected to decrease with shelf-life time.

Two different behaviours were observed between samples with (ZC_CT and ZC_ZC) and without (CT_CT and CT_ZC) pre-harvest zinc application. In ZC_CT and ZC_ZC samples the ratio between chlorophyll a and chlorophyll b increased abruptly during the first 3 days, after which it decreased, as was expected due to faster chlorophyll *b* degradation (Figure 23B), compared to chlorophyll *a* (Figure 23A). On the other hand, in CT_CT and CT_ZC samples this ratio increased during 8 days, indicating faster chlorophyll *b* degradation during this period, compared to chlorophyll *a*. However, this behaviour is not observed in chlorophyll *a* and chlorophyll *b* content evolution during shelf-life (Figure 23). After 8 days of shelf-life, the ratio between chlorophyll *a* and *b* there was a decrease in this parameter.

The changes in the ration between chlorophyll *a* and *b* are mainly due to chlorophyll *b* variation during shelf-life, since chlorophyll *a* content remained fairly constant. As a result, it can be assumed that chlorophyll *b* is most influent to color perception of in pre-harvest foliar zinc treatment (Table D1, Appendix D).

The variation of carotenoid content during shelf-life in lettuce homogenate samples is shown in Figure 26.

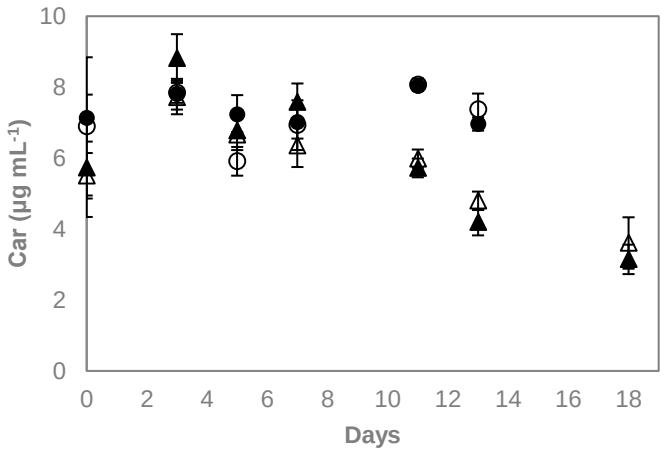


Figure 26 – Evolution of carotenoid content during shelf-life of lettuce homogenates. ● - CT_CT; ○- CT_ZC; ▲ - ZC_CT; △ - ZC_ZC. Error bars are standard deviation (*n*=3).

Carotenoid content was maintained in treated samples during 7 days, in all samples. After 7 days, ZC_CT and ZC_ZC samples showed a slightly steeper decrease than remaining samples. Overall, pre-harvest zinc treatments did not have a strong effect on carotenoid content of lettuce homogenates.

4.7. Relationship between color and chlorophylls

As previously discussed (section 2.5), although chlorophyll pigments determine the green color of fruit and vegetable matrices, the relationship between chlorophyll content and color is not always evident. We explored the relationship between chlorophyll content and hue angle in the dataset of the vegetable homogenates produced from the four raw materials in study. Linear regression between chlorophyll content (µg mL^{-1}) and hue angle value for spinach, parsley, broccoli and lettuce samples, during shelf-life is represented in Figure 27.

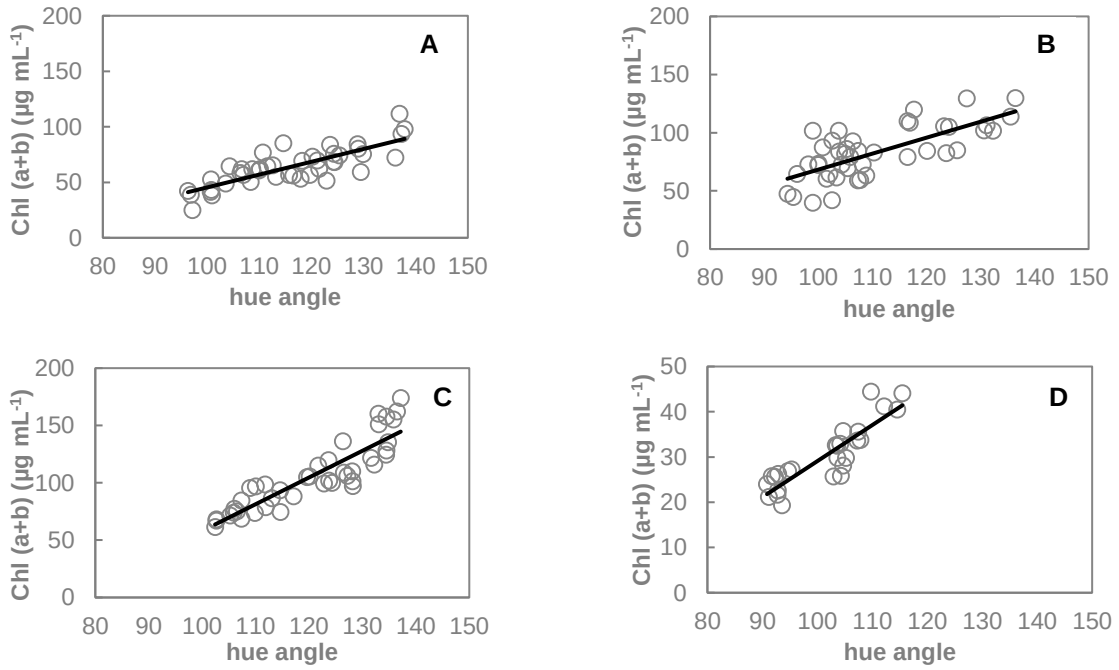


Figure 27 – Correlation between total chlorophyll content and hue angle of spinach (A), parsley (B), broccoli (C) and lettuce (D) homogenate samples. Regression equations, correlation (r) and sample size (N): (A)Y=1.2X – 70.2, r=0.8, *n*=43; (B) Y=1.4X – 68.9, r=0.7, *n* =43; (C) Y=2.3X – 179.5, r=0.9, *n* =42; (d) Y=0.8X – 50.6, r=0.9, *n* =26.

Significant positive linear correlations (*P*≤0.05) were observed between chlorophyll contents and sample hue: 0.9 for lettuce and broccoli samples, 0.8 for spinach samples and 0.7 for parsley samples. Despite the overall statistical association between hue angle and chlorophyll content, the variability observed in the data accouts for the lack of coincidence between hue and chlorophyll, as previous reported. For example, in spinach, the same chrophyll content of 51.4 and 52.5 µg mL^{-1} corresponded to hue angles of 122.9° and 107°, respectively, which are very distinct hues angle values. Similarly in parsley, small differences in chlorophyll, e.g., 84.7 and 83.6 µg mL^{-1}, corresponded to very different hue angles, 125.7° and 103.9°, respectively.

In contrast with reports in the literature (Sinnecker et al., 2002; Gonçalves et al., 2009; Martins & Silva, 2002), no significant relation between the instrumental a* value and chlorophyll content or total color difference and chlorophyll content, was observed (data not shown).

Chapter 5

Final Remarks

This chapter presents the main conclusions drawn from the studies and suggests future work to address research questions that were raised or remained answered.

5.1. Conclusions

Chlorophyll and green color stabilization in vegetable homogenates produced from freshly harvested leaves were dependent on raw material, pre-harvest treatments, pH, and the presence of the food additives zinc chloride and sodium bicarbonate.

Higher concentrations of chlorophyll in raw materials can lead to darker or more intense green color of produced homogenate, and help maintain visual green color of pasteurized samples but does not necessarily affect stability. Broccoli had the highest total chlorophyll content as well as lower losses during pasteurization, followed by spinach, parsley and lettuce. Spinach homogenates had the second highest chlorophyll content but suffered the higher chlorophyll loss during pasteurization. Heat treatment also induced a decrease of visual green color intensity as well as hue value. Although spinach, parsley and broccoli leaves had all different initial chlorophyll concentrations and different chlorophyll losses during pasteurization, no visually perceived color differences were detected in these samples after the heat treatment.

The effect of food additives was dependent of pH and raw material. At pH 8.5 and 6.0 sodium bicarbonate increased visually perceived color intensity and corresponding hue of samples more efficiently than zinc chloride, although both had a positive effect on sample color characteristics when compared to control samples. At pH 4.5, the additives did not help color maintenance in the homogenates from any of the raw materials. While chlorophyll loss was lower in broccoli and parsley homogenates containing zinc chloride or sodium bicarbonate, no significant improvement was observed in spinach, irrespective of pH.

Homogenate pH values shifted during shelf-life towards the characteristic raw material pH. All homogenate samples had a similar behaviour during the first 2 d of shelf-life, with initial hue and chlorophyll stabilization, followed by a decrease in both parameters. Sodium bicarbonate had a greater effect on maintenance of hue, with lower chlorophyll loss during shelf-life when compared to the treatment with zinc chloride, irrespective of pH and plant material.

Pre-harvest foliar application of zinc chloride can help improve chlorophyll retention, inducing higher hue values and higher chlorophyll content on processed vegetable raw materials. Samples without foliar treatments had lower initial hue and had a faster browning after the 2nd day of shelf-life, when compared to samples with foliar treatment. Visual color of samples with pre-harvest treatment was darker than that of samples without treatment, and showed higher maintenance during shelf-life. The addition of zinc chloride as a food additive to the homogenate allowed a slight increase in hue, although visually this was not perceived.

5.2. Future Work

Harnessing the green color of leafy vegetables on microbial safe processed foods and beverages without food coloring agents remains a challenge.

Our study has shown significant differences in color stability among four raw materials used to prepare the homogenates. Further studies on the interaction of the chlorophyll molecules and the matrix are required to explain why chlorophyll from some species are less heat-labile and more stable than others.

A better understanding of the relationship between color and chlorophyll content is needed. An objective quality control measure of green color is useful for product development and quality control in industrial settings.

Although the influence of sodium bicarbonate on green color stability is widely known, the chemistry of sodium chlorophyllin complexes and its formation deserve further studies, as do the differential effects of pH and the sodium or bicarbonate ions. A better understanding of

the mechanisms involved in chlorophyll-sodium bicarbonate reactions would allow the optimization of the conditions and variables involved.

The high-temperature-short-time pasteurization or sterilization treatments were not addressed in our study. Since green color is more stable under alkaline conditions, the effect of sterilizing condition required to assure microbial safety must be assessed. If successful this would allow microbial stabilization of vegetable food products, without sacrificing color of final product.

Our results also suggest that pre-harvest treatments with zinc ions can improve color of processed products as well as chlorophyll resistance to heat treatments. Additional studies are required to develop this proof-of-concept and generalize it to different leafy vegetables and optimization of the protocol of raw material production.

In order to obtain processed vegetable food products, with stable and noticible green color, many processing parameters need to be optimized, such as processing time and temperature, pH, type of raw material and desired final matrix (e.g. liquid, puree) and packaging. The influence of other variables, such as the influence of light and oxygen concentration inside the packaging should also be addressed, in order to understand their influence on chlorophyll content and green color of processed products.

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Appendix

Appendix A – Effect of raw material, pH and food additives on soluble solid content and color parameters (L* and C*) of vegetable homogenates without (NHT) and with (HT) heat treatment and during shelf-life

Table A1 – Effect of sodium bicarbonate and zinc chloride on soluble solid content of samples without (NHT) and with (HT) heat treatment. Values are means ± s.d. (*n*=3). Means followed by a different letter are significantly different according to Fisher's LSD test at α=0.05 (within columns).

Salt and pH treatment	Heat Treatment	Spinach	Parsley	Broccoli
CT	NHT	0.5^{ed}±0.1	0.8^{c}±0.1	0.8^{f}±0.1
	HT	0.5^{ed}±0.2	0.2^{g}±0.1	0.7^{f}±0.1
SB_4.5	NHT	0.9^{ba}±0.1	1.0^{b}±0.1	1.1^{e}±0.1
	HT	0.7^{cb}±0.1	0.7^{e}±0.1	1.3^{c}±0.1
SB_6	NHT	0.9^{ba}±0.1	1.3^{a}±0.1	1.2^{cd}±0.1
	HT	0.7^{cb}±0.1	0.7^{ed}±0.1	1.2^{de}±0.1
SB_8.5	NHT	0.9^{ba}±0.1	1.2^{a}±0.1	1.4^{b}±0.1
	HT	1.0^{a}±0.1	0.7^{ed}±0.1	1.5^{a}±0.1
ZN_4.5	NHT	0.3^{e}±0.1	0.6^{f}±0.1	0.6^{h}±0.1
	HT	0.3^{e}±0.1	0.2^{g}±0.1	0.7^{fgh}±0.1
ZN_6	NHT	0.4^{e}±0.1	0.8^{dc}±0.1	0.6^{gh}±0.1
	HT	0.6^{d}±0.2	0.2^{g}±0.1	0.6^{gh}±0.1
ZN_8.5	NHT	0.4^{e}±0.1	0.7^{e}±0.1	0.7^{f}±0.1
	HT	0.3^{e}±0.1	0.2^{g}±0.1	0.7^{fg}±0.1

Table A2- – Effect of sodium bicarbonate and zinc chloride on color parameters L* and C* of samples without (NHT) and with (HT) heat treatment. Values are means ± s.d. (*n*=3). Means followed by a different letter are significantly different according to Fisher's LSD test at α=0.05 (within columns).

Salt and pH treatment	Heat treatment	Spinach		Parsley		Broccoli	
		L*	**C***	**L***	**C***	**L***	**C***
CT	NHT	26.0^{b}±0.1	7.3^{c}±0.3	26.0^{h}±0.1	7.5^{i}±0.4	27.2^{f}±0.2	9.4^{f}±0.3
	HT	22.1^{cd}±0.1	7.3^{c}±0.1	33.2^{a}±0.2	16.3^{ab}±1.0	33.8^{ab}±0.1	16.8^{a}±0.2
SB_4.5	NHT	26.3^{b}±0.8	8.9^{ab}±0.6	27.9^{e}±0.2	10.2^{f}±0.2	29.4^{d}±0.1	12.7^{d}±0.4
	HT	25.1^{cd}±0.3	6.0^{d}±0.1	31.0^{c}±0.2	11.5^{e}±0.3	33.7^{ab}±0.6	13.3^{d}±1.3
SB_6.0	NHT	26.0^{c}±0.3	7.5^{c}±0.3	26.1^{h}±0.1	7.8^{hi}±0.2	25.9^{g}±0.8	8.1^{g}±0.4
	HT	24.2^{e}±0.6	7.2^{c}±0.4	30.6^{c}±0.4	12.6^{d}±0.2	31.1^{c}±0.1	14.2^{c}±0.5
SB_8.5	NHT	25.0^{d}±0.3	6.0^{d}±0.3	26.2^{gh}±0.1	7.6^{i}±0.2	26.4^{g}±0.1	8.1^{g}±0.4
	HT	22.4^{f}±0.4	5.4^{e}±0.1	29.6^{d}±0.5	13.8^{c}±1.0	28.0^{e}±0.1	10.5^{e}±0.1
ZN_4.5	NHT	25.3^{a}±0.2	8.9^{a}±0.3	26.9^{fg}±1.0	10.5^{f}±0.5	27.6^{ef}±0.1	9.9^{ef}±0.3
	HT	26.6^{b}±0.3	7.5^{c}±0.3	33.8^{a}±0.5	15.9^{b}±0.8	34.2^{a}±0.1	15.2^{b}±0.1
ZN_6.0	NHT	24.6^{de}±0.9	8.7^{ab}±0.7	25.9^{h}±1.1	8.4^{gh}±0.5	26.2^{g}±0.8	8.6^{g}±0.4
	HT	25.3^{c}±0.1	7.4^{c}±0.5	33.8^{a}±0.3	10.9^{a}±0.3	33.6^{b}±0.3	16.8^{a}±0.2
ZN_8.5	NHT	24.1^{e}±0.5	8.4^{b}±0.1	27.4^{ef}±0.2	8.8^{g}±0.3	26.1^{g}±0.1	8.0^{g}±0.4
	HT	24.3^{e}±0.3	7.0^{c}±0.3	31.8^{b}±0.3	16.1^{b}±0.4	31.4^{c}±0.1	15.5^{b}±0.2

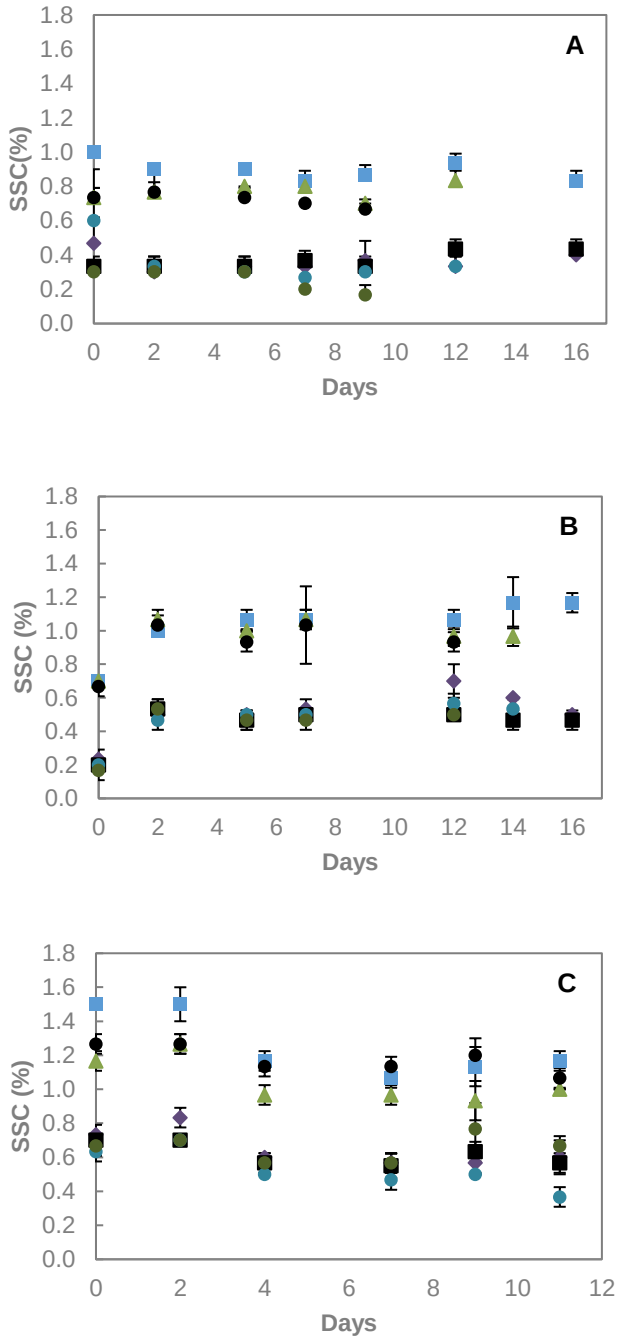


Figure A1 – Soluble solid content evolution during shelf-life for spinach (A), parsley (B) and broccoli (C) homogenate; ◆- CT; ■- SB_8.5; ▲- SB_6; ● - SB_4.5; ■ - ZC_8.5; ● - ZC_6; ● - ZC_4.5. Error bars are standard deviation (*n*=3).

Appendix B – Effect type of raw material, pH and food additives on carotenoid content of vegetable homogenates without (NHT) and with (HT) heat treatment and during shelf-life

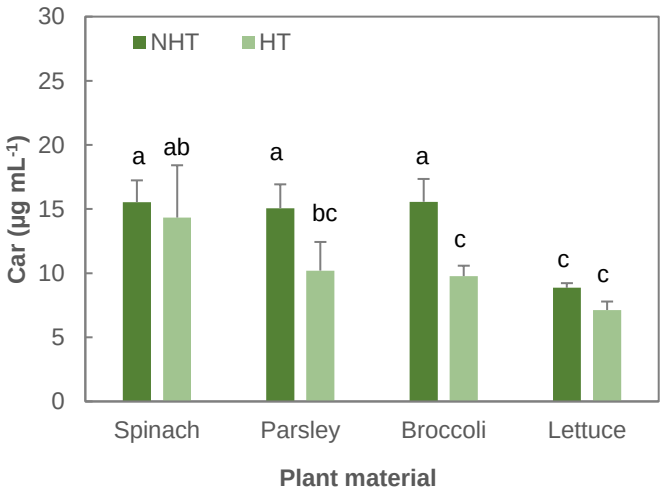


Figure B1 - Heat treatment effect on carotenoid content of vegetable homogenates from different plant materials. NHT – No heat treatment; HT- heat treatment. Error bars are standard deviation (*n*=3). Means followed by a different letter are significantly different according to Fisher's LSD test at α=0.05.

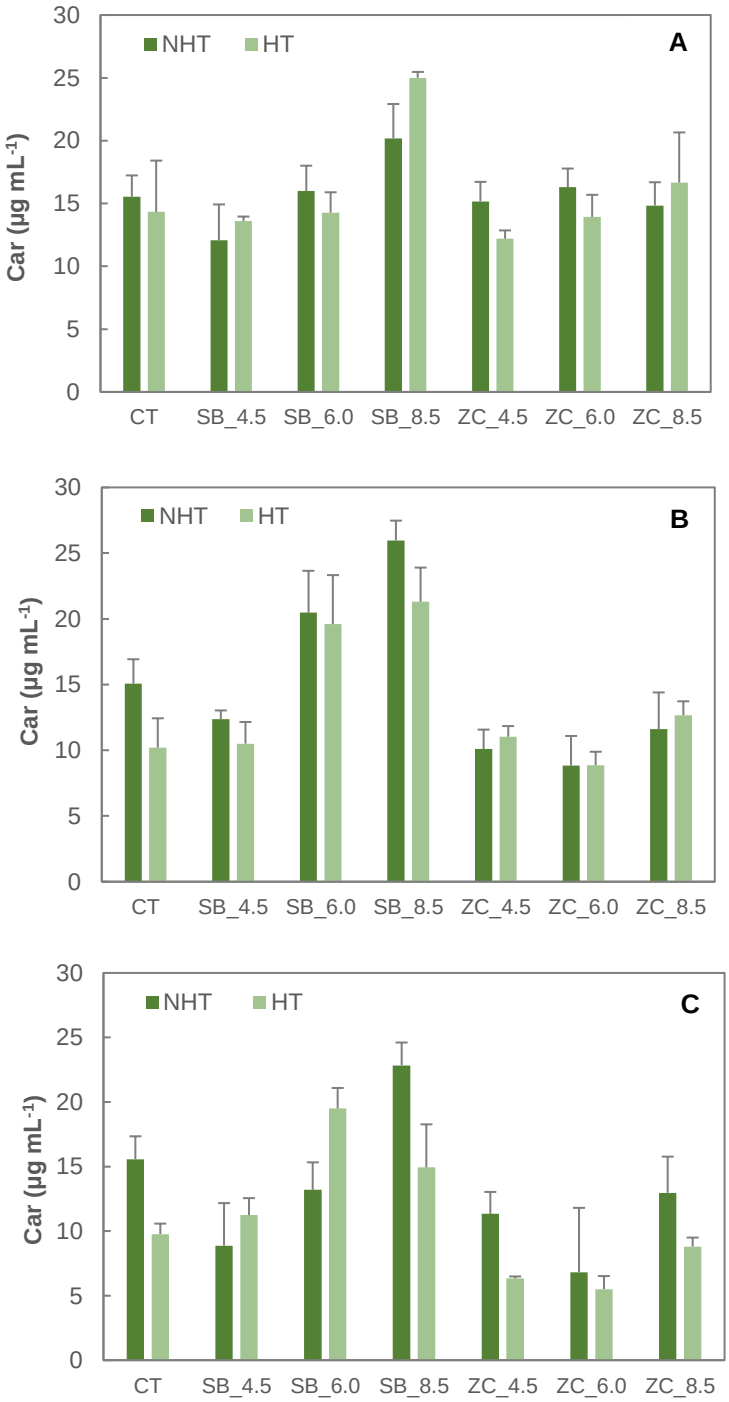


Figure B2 - Heat treatment, pH and food additive effect on carotenoid content of vegetable homogenates: spinach (a), parsley (b) and broccoli (c). Error bars are standard deviation (*n*=3). Means followed by a different letter are significantly different according to Fisher's LSD test at α=0.05.

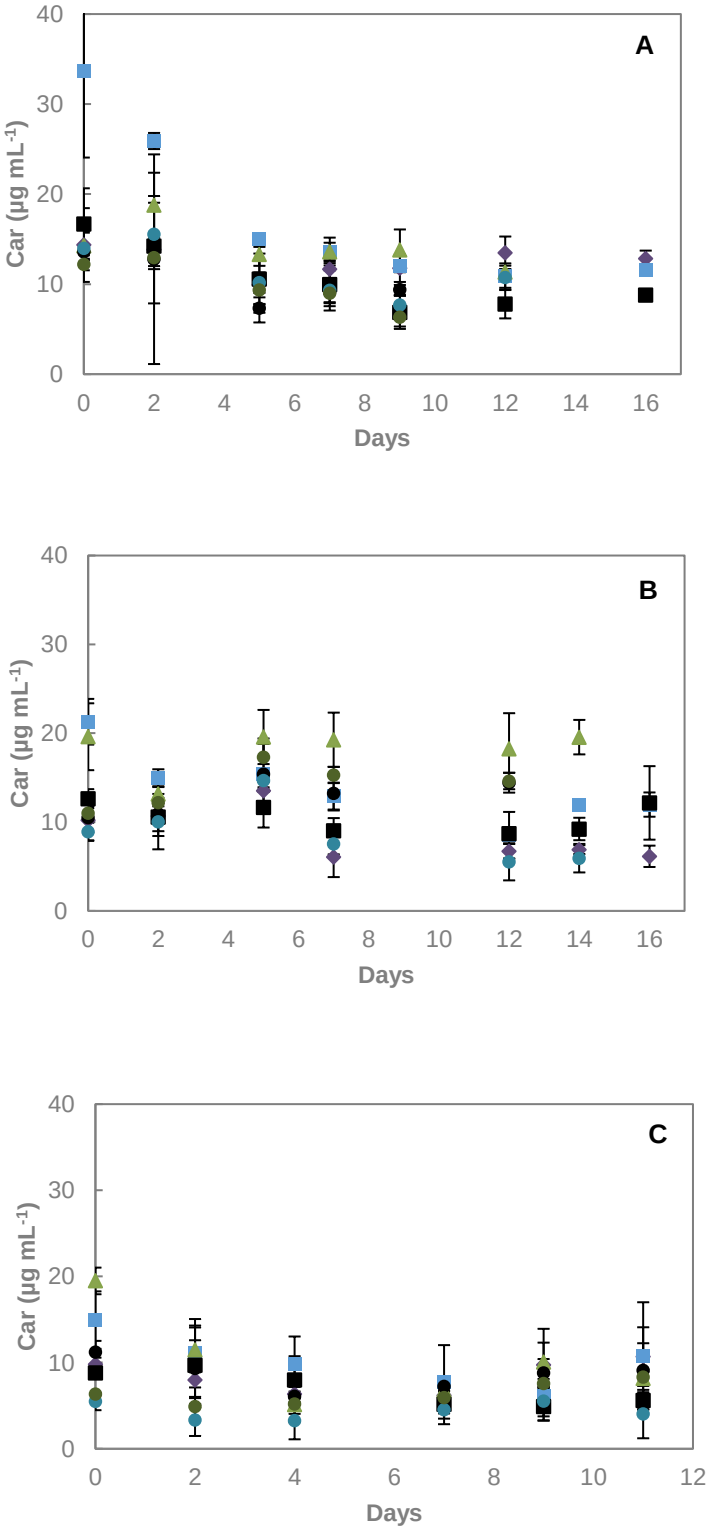


Figure B3 - Carotenoid evolution during shelf-life for spinach (A), parsley (B) and broccoli (C) homogenates. ◆- CT; ■- SB_8.5; ▲- SB_6; ● - SB_4.5; ■ - ZC_8.5; ● - ZC_6; ● - ZC_4.5. Error bars are standard deviation (*n*=3).

Appendix C – Effect of raw material, pH and food additives on visually perceived color of pasteurized vegetable homogenates during shelf-life

Table C1 – Effect of pH and food additives on visually perceived color of pasteurized spinach homogenates during shelf-life.

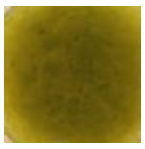
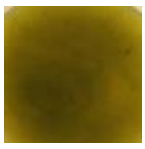
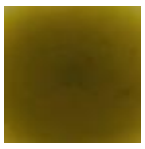
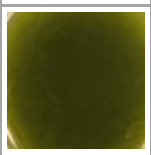
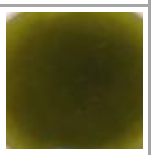
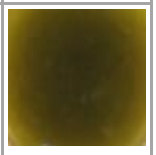
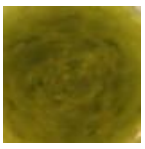
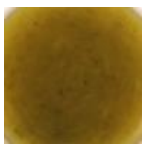
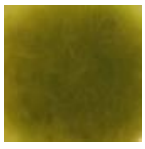
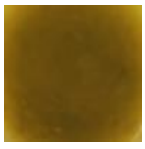
	Shelf-Life (Days)						
	0	**2**	**5**	**7**	**9**	**12**	**16**
CT							
SB_8.5							
SB_6.0							
SB_4.5							
ZC_8.5							
ZC_6.0							
ZC_4.5							

Table C2 – Effect of pH and food additives on visually perceived color of pasteurized parsley homogenates during shelf-life.

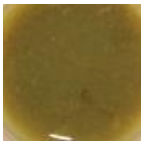
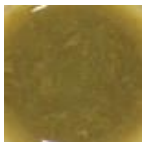
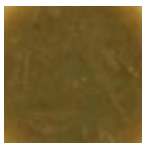
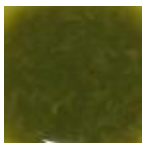
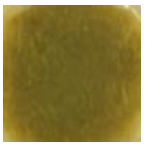
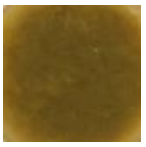
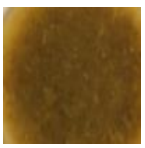
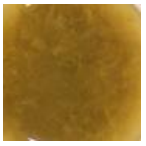
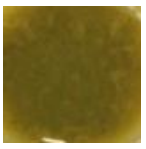
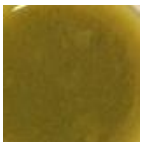
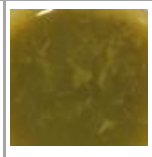
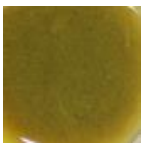
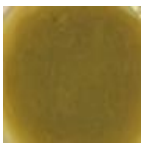
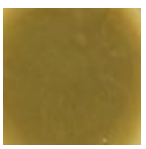
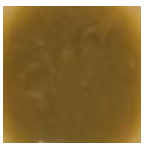
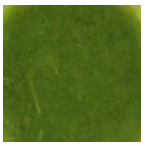
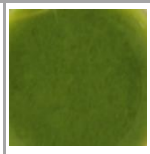
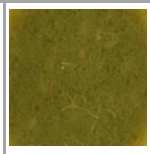
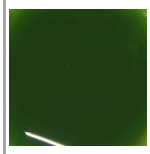
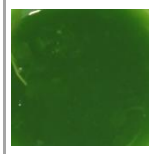
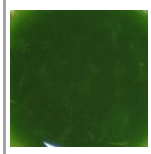
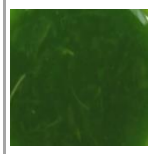
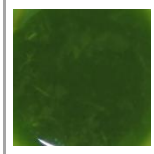
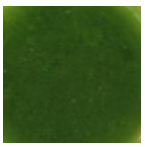
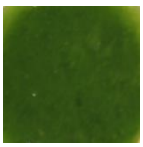
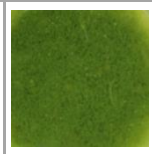
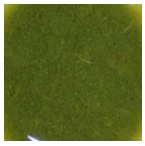
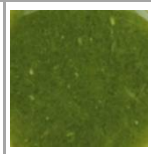
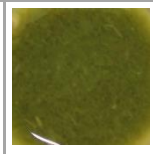
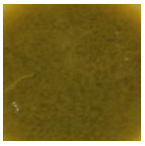
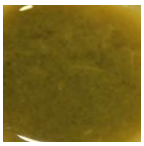
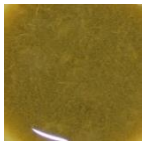
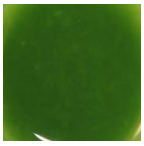
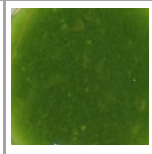
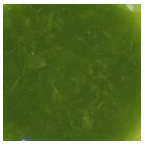
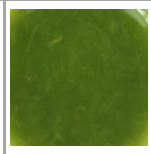
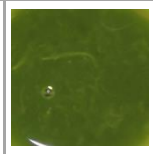
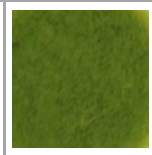
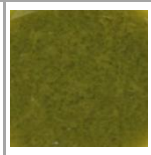
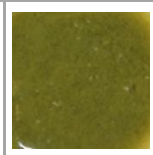
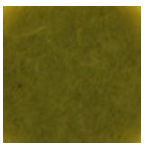
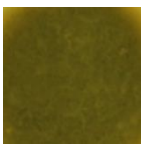
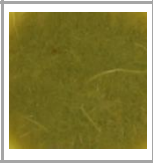
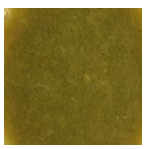
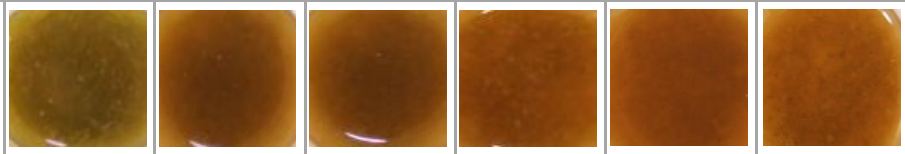
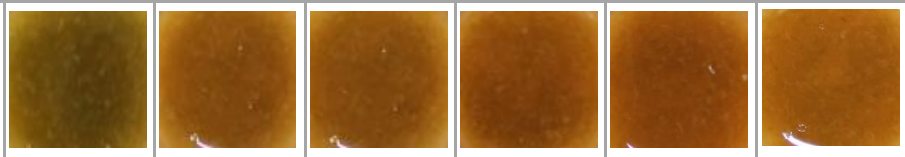
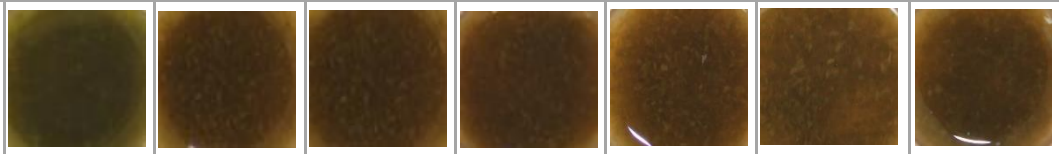
	Shelf-Life (Days)						
	0	**2**	**5**	**7**	**12**	**14**	**16**
CT							
SB_8.5							
SB_6.0							
SB_4.5							
ZC_8.5							
ZC_6.0							
ZC_4.5							

Table C3 – Effect of pH and food additives on visually perceived color of pasteurized broccoli homogenates during shelf-life.

	Shelf-Life (Days)					
	0	**2**	**4**	**7**	**9**	**11**
CT						
SB_8.5						
SB_6.0						
SB_4.5						
ZC_8.5						
ZC_6.0						
ZC_4.5						

Appendix D – Effect of foliar application treatments and food additives on visually perceived color of pasteurized lettuce homogenates during shelf-life

Table D1 – Effect of pH and food additives on visually perceived color of pasteurized lettuce homogenates during shelf-life.

Shelf-Life (Days)							
	0	**3**	**5**	**7**	**11**	**13**	**18**
CT_CT							
CT_ZC							
ZC_CT							
ZC_ZC